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NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

ANNUAL REPORTS

INTRAMURAL RESEARCH PROGRAMS

OCTOBER 1, 1986 TO SEPTEMBER 30, 1987

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PREFACE

The National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) is responsible for research on a broad range of diseases and abnormal conditions that at one time or another effect almost every family in the Nation. These diseases are usually long-lasting and may be severely disabling. They generate a tremendous burden of human suffering and economic loss, both to the individual and to society.

In its extramural research programs, the Institute supports a wide spectrum of basic and clinical research, and research training, in the fields of rheumatology, muscle biology, orthopaedics, bone and mineral metabolism, and dermatology.

Major avenues of arthritis research include the roles of humoral and cellular immune abnormalities in the pathogenesis of rheumatic disease; mechanisms by which mediators of inflammation, such as the prostaglandins and leukotrienes, promote and regulate inflammatory reactions in arthritis; immunogenetic factors underlying susceptibility to arthritis; ultra-structure and pathophysiology of collagens, proteoglycans, and other components of connective tissue; and metabolic derangements underlying osteoarthritis and gout/pseudogout. Musculoskeletal disease research includes studies of the basic structure of bone and cartilage; metabolic bone disease; fractures and the healing process; prostheses and relevant biomaterials; biomechanics and bioelectric phenomena; bone transplantation; the physiology of normal and abnormal gait; and inherited connective tissue

diseases. In dermatology, investigations are under way on a wide range of problems including psoriasis, acne, vitiligo, atopic dermatitis, and fatal blistering "bullous" diseases. Employing new laboratory techniques, scientists are attempting to clarify the roles of immunopathology, phototoxicity, and metabolic abnormalities in the etiology of these cutaneous disorders.

The Intramural Research Program of the Institute consists of two major investigation units: the Arthritis and Rheumatism Branch (ARB) and the Laboratory of Physical Biology (LPB). Dr. Henry Metzger, an internationally-renowned immunologist, was appointed this past year as Scientific Director of NIAMS. Also, this past year, Dr. John H. Klippel, a highly talented clinical investigator of rheumatic diseases, was appointed the Institute's Clinical Director.

The Arthritis and Rheumatism Branch was established over 30 years ago as a major intramural component of the National Institute of Arthritis and Metabolic Diseases. It has been one of the outstanding centers for laboratory and clinical research and training in rheumatology, basic immunology and related areas. Research in the Branch is directed by five Senior Investigators and covers a broad spectrum of disciplines: biochemistry, molecular biology, genetics, cell biology, immunology, pathology, pharmacology , and clinical investigations. In addition to collaborations within the Branch, there are many cooperative projects with other groups at the National Institutes of Health.

The Branch has been organized into three research sections of Clinical Immunology, Cellular Immunology and Connective Tissue Diseases. This year we have recently added to the Branch an Orthopaedic Research Unit. This area has great promise for research productivity in the future.

The other major component of the NIAMS Intramural Program is the Laboratory of Physical Biology (LPB), the underlying scientific disciplines of which are biophysics and structural biology. The Laboratory studies biological problems using a broad range of physical techniques. They include X-ray diffraction, high resolution mechanical recording electron microscopy, image processing, surface film techniques, and radiation inactiviation analysis. The Laboratory consists of three Sections.

In the Section on Muscle Biophysics, mechanisms of muscle regulation and contraction are studied, using both structural and functional techniques. A unique capability of the Laboratory is to bring to bear both structural (X-ray diffraction, electron microscopy) and functional techniques (force and displacement recording, radiation inactivation analysis) on specific questions in muscle physiology.

In the Section on Macromolecular Biophysics, lipid dispersions in water are investigated as models of the structure and physical states of lipids in cell membranes. The possible physicochemical relationship between diseased states in tissues and membrane bilayer instability is

examined. Actions of ionizing radiation on macromolecules are used to determine the in vivo size of the functional units for different biological activities (hormone receptors, enzyme complexes, and regulatory units).

In the Section on Structural Biology, mechanisms of macromolecular assembly and structure-function relationships at the supramolecular level are investigated by high resolution microscopy in conjunction with digital image processing and by cryo-electron microscopy. Computer modelling is also used to integrate data from other biochemical and biohysical sources. Systems of interest include the structure and assembly of viruses, intermediate filaments including keratin filaments, clathrin and coated membranes, and other fibrous polymers and oligomeric protein complexes.

The Institute takes great pride in the scientific achievements of its intramural scientists and in their high repute within the scientific community.

Lawrence E. Shulman, M.D., Ph.D.
Director
National Institute of Arthritis and
Musculoskeletal and Skin Diseases

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NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

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ANNUAL REPORT
LABORATORY OF PHYSICAL BIOLOGY
NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

Muscle Contraction

This year the kinetic properties and structure of the weakly binding actomyosin state, seen when ATP is bound to myosin, were further characterized in rabbit muscle fibers. In addition, a new mechanism was discovered by which muscle fibers in the rigor state (ATP-free) could produce force when the filament lattice is subjected to radial expansion. The physiological role of the megadalton proteins, titin and nebulin, was studied by correlating mechanical properties of resting fibers with A-band movement in activated fibers. Finally, the recently reported involvement of nebulin in Duchenne muscular dystrophy was reexamined.

Kinetic properties of the weakly binding actomyosin crossbridge.

The myosin state seen when ATP binds to the active site in the absence of Ca^{2+}, (MATP), shows rapid, reversible, attachment and detachment to actin, with detachment rate-constants in the neighborhood of 5×10^3 s^{-1}. Previous experiments with skinned muscle fibers were performed at very low ionic strength in order to maximize the mount of time a weakly-binding myosin head would spend attached to actin. An important question that remained following these studies was how much time the heads spend attached to actin at physiological ionic strength. It was found that the crossbridge detachment rate-constants are not greatly affected by ionic strength and that the percentage of time an MATP head spends attached to actin decreases about 10-fold as ionic strength is increased from very low values to physiological values. This implies that the main effect of increasing ionic strength is to cause a large decrease in the crossbridge attachment rate-constants. Since the myosin head with ATP bound spends 50-100% of its time attached to actin at low ionic strength, at high ionic strength it spends 5-10% of its time attached to actin. The demonstration that myosin heads attach to actin at all ionic strengths suggests that, as was shown previously at low ionic strength, muscle relaxation at normal ionic strength is caused primarily by blockage of a step subsequent to attachment, and not by blockage of the attachment step. (Schoenberg)

Crossbridge structures at low ionic strength.

Experiments with relaxed and activated fibers were carried out at the synchrotron radiation facility at Hamburg (DESY). The X-ray beam at DESY is as much as 1000 times more intense than conventional laboratory sources, which allows exposure to be correspondingly shorter. Using single skinned rabbit psoas fibers at 50 mM ionic strength, upon activation changes occur only in the intensity of the first reflection, with that of the second reflection remaining unchanged. This change in I_{10} could not simply be due to a change in the number of attached crossbridges in the relaxed configuration, since this would cause a greater change in I_{11} rather than in I_{10}. Based on model calculations, the greater change in I_{10} probably represents a radial movement of myosin mass away from the thick filament backbone, but no significant increase in the mass surrounding the thin filament. Assuming that attached

crossbridges in relaxed fibers are in a state prior to the "power-stroke" in the crossbridge cycle, the present results suggest that the power-stroke consists of a configurational change in the part of the attached myosin head that is distal to the actin filament. (Yu, Brenner)

Crossbridge rate constants in fibers and in solution.

It has been postulated that an unstrained myosin head should detach in a fiber with about the same rate-constant as myosin subfragment-1 (S1) detaches from actin in solution. In the presence of the ATP analogue magnesium pyrophosphate, myosin heads in fibers detach not with a single rate-constant but with a wide range of detachment rate-constants. The fastest of these rate-constants are comparable to the detachment rate-constant of S1 from actin in solution, but many of the crossbridges detach with rate-constants perhaps 100-fold slower than the S1 detachment rate-constant. While examining how the rate-constant for crossbridge detachment in the presence of MgPPi depends upon MgPPi concentration, the concentration dependence relationship was found to be sigmoidal. The most likely explanation for this is that both of the S1 heads of a crossbridge need to bind MgPPi before either has a high probability of relaxing a substantial fraction of the tension it supports. This in turn provides an explanation for why the crossbridge detachment rate-constants in the fiber are smaller than the corresponding rate-constant in solution. If both heads of a crossbridge must detach simultaneously before tension is reduced, this process would indeed be expected to be slower than the rate at which S1 detaches from actin in solution. (Schoenberg)

Force production by rigor fibers subjected to lateral expansion.

It has been known for many years that rigor muscle fibers develop considerable force when the myofilament lattice spacing is made to increase by lowering the ionic strength and/or raising the pH. This effect has been explained in many ways, including an influence of lattice spacing on axial crossbridge length. However, electron microscopy showed that lattice expansion is associated with the separation of the thick, myosin-containing filaments into three subfilaments in the peripheral parts of the A-band. Force appears to be produced by the decrease in axial length of the subfilaments resulting from thick filament dissociation. A direct crossbridge effect seems to be ruled out because the lateral distance between the actin filament and the myosin subfilaments was nearly independent of the myofilament lattice spacing. (Barbosa, Podolsky)

Physiological role of titin and nebulin.

Radiation inactivation analysis of muscle fibers activated at sarcomere length of 2.6 μm showed that the thick filaments are mechanically linked to the nearest Z disc by elastic filaments made of titin and/or nebulin. Similar experiments carried out at a shorter sarcomere length, 1.6 μm, where positioning of the thick filaments by elastic filaments would not be expected to affect force development, gave a somewhat smaller target size for force development but one that was still in the megadalton range. This unexpected result suggests that titin and/or nebulin may directly affect the actomyosin crossbridges as well as order the thick filaments in activated muscle fibers. (Horowits, Kempner, Podolsky)

Muscular dystrophy.

Muscle biopsies were taken from patients with Duchenne muscular dystrophy (DMD) and individual muscle fibers were assayed for contractile force and muscle protein content. The average muscle force/unit area in DMD fibers was slightly less than control value, but protein content (including titin and nebulin) was almost the same in DMD and in normal fibers. The results suggest that the nebulin content of muscle fibers is not controlled by the DMD gene, and that the disease may be caused primarily by a decrease in the number of muscle fibers in a given muscle. (Horowits, Dalakas (NINCDS), Podolsky)

High Resolution Electron Microscopy

Tail fibers of bacteriophage T7.

The T7 tail-fiber represents an experimentally tractable example of a viral receptor-recognition protein. Piece-wise correlation averaging of electron micrographs of molecules prepared both by negative staining and by freeze-drying without staining, was combined with predictions based on the known sequence of the gp17 protein (553 residues), to formulate a detailed model of this fibrous protein. The fiber is a trimer and consists of three domains. The proximal and distal half-fibers are both rods ~ 16nm long joined at a kink angle of ~ 90° and are linked to the virion by the third domain. Of particular interest is the proximal half-fiber which is a triple-stranded coiled-coil of α-helices, one of the few well-authenticated examples of this conformation. (Steven)

Physiological states of vertebrate skeletal muscle visualized in frozen-hydrated thin sections.

Conventional approaches to visualization of the force-generating interactions between actin and myosin filaments in the myofilament lattice have been based on X-ray fiber diffraction and thin section electron microscopy. However both techniques have their limitations. The X-ray diffraction experiments provide sets of Fourier amplitudes from "physiological" material, but lack the phase information required to completely specify the corresponding electron density maps. With conventional thin sectioning methods, the "nativeness" of the muscle structure thus represented is suspect. The recently developed technique of cryo-electron microscopy has the potential for direct imaging of preparations maintained in their native state. Image processing methods have been devised to compensate for the adverse effects of non-linear contrast transfer (incurred by the de-focussed bright-field imaging used in this type of electron microscopy), and for the various kinds of spatial disordering to which frozen-hydrated sections are particularly susceptible. The results reveal differences between the distributions of density in muscle in the relaxed and rigor states and provide phase information for the equatorial X-ray reflections. (Steven, Trus (DCRT), McDowall (EMBL), Barbosa, Podolsky)

Intramural AIDS program.

The Section on Structural Biology is participating in the intramural program designed to bring the experimental methods of modern biology to bear on the molecular constituents of the Human Immunodeficiency Virus (HIV), the causative agent of AIDS. The plan is to apply to gp160 and gp120, the surface glycoproteins of HIV, the methods of structural analysis which have been developed in work on the corresponding protein of phage T7 (cf. above). A structural map of HIV proteins, even at relatively modest resolution, may be of use in vaccine design, and perhaps also as an aid in early stages of high resolution structure determination by X-ray crystallography. (Steven)

Cell Membranes

Mechanism of cell membrane assembly and disassembly.

These studies have focussed on processes which form membrane lipid bilayers, and in particular on the conditions which lead to the assembly of the unilamellar or single bilayer state. Membrane lipids assemble spontaneously *in situ* into the unilamellar state at the growth temperature of the cell; the composition of the membrane is determined by the lipids that are present in the metabolic pool of the cell. The growth temperature is a critical point T* at which the unilamellar state is optimally stable. At temperatures above and below T* non-unilamellar lipid structures form. Thus a critical temperature and composition are required for forming the unilamellar membrane state. According to this view of the membrane structure, if the ambient temperature of the cell deviates from T* the membrane must shift from the normal unilamellar to a multibilayer state. This model for membrane disassembly raises an important issue concerning cell stability, since most cell preparations appear to be viable over a relatively wide temperature range. This question has now been examined in several systems and a partial understanding of how membrane bilayers respond to thermal fluctuations has been reached. For poikilotherms, such as bacteria, it has been shown that the cells accommodate to growth temperature shifts by synthesizing new membrane lipid whose composition conforms to the assembly requirements of the new T*. However, for most homeotherms which cannot accommodate to new ambient temperatures by synthesizing new lipid, the cells become leaky when temperatures are elevated above T*. The concept of the critical bilayer state is currently being examined in the context of possibly providing an explanation for membrane failure in diseased states. Under study at present are lipid extracts from the cortex and cerebellum of deceased Alzheimer patients. Lesions are generally localized to the cortex. In preparation for these studies control experiments were done to test whether lipids from a tissue as complex as brain exhibit a critical temperature. Preliminary studies with lipid extracts of rat cerebellum, cortex, and brain stem indicate a critical temperature for the formation of the unilamellar state in each of these tissues very close to body temperature. (Gershfeld)

Platelet Aggregation

Human platelets aggregate spontaneously when decompressed to 1/3 atmosphere. This process is inhibited by 10mM menthol and menthone and 5 μm thymol, an aromatic derivative of menthol, and is fibrinogen dependent. Increasing the concentration of neutral salts can inhibit platelet aggregation. Molecules having a large electric dipole moment may inhibit platelet aggregation by a similar mechanism. A tetrapeptide having the amino acid sequence RGDS (Arg-Gly-Asp-Ser) is found in both the specific adhesive site on the membrane surface of platelets as well as on the alpha-chain of fibrinogen. The synthetic tetrapeptide RGDS was found to inhibit aggregation, which supports the idea that these residues may be specifically involved in aggregation. (Murayama)

Radiation Inactivation Analysis

Target analysis of radiation inactivation had three aspects. (1) Theoretical studies developed a target analysis for complex systems which show a radiation-induced change in intrinsic activity (K_m for enzymes, K_D for receptors). (2) Fundamental studies of radiation action which showed the same temperature effect previously found for enzymes also holds for synthetic polymers, and found that the phenomenon is due to the temperature-dependence of free radical generation. Other fundamental studies proved that radiation action on the oligosaccharide portion of glycoproteins did not contribute to the target size, and this was due to the absorption of the radiation energy near the primary ionization. (3) The method was applied to several new biochemical systems, and most notably to a complex physiological system, muscle contraction (cf. above). (Kempner)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 27000-25 LPB

PERIOD COVERED October 1, 1986 through September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Mechanism of Muscular Contraction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard J. Podolsky	Chief	LPB/NIAMS
Others:	Leepo C. Yu	Research Physicist	LPB/NIAMS
	Robert Horowits	NRSA	LPB/NIAMS
	Maria Barbosa	NRSA	LPB/NIAMS

COOPERATING UNITS *(if any)* Dr. Alasdair Steven, Section on Structural Biology, LPB; Dr. Ellis Kempner, Section on Macromolecular Biophysics, LPB; Dr. Alasdair McDowall, European Molecular Biology Laboratory, Heidelberg, W. Germany; Dr. Marinos Dalakas, NINCDS.

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Muscle Biophysics

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Contractile force in muscle fibers is generated by crossbridges that form between the myosin-containing and actin-containing filaments and hydrolyze ATP. During shortening, the two types of filaments slide relative to each other. Using skinned fiber preparations from rabbit, structural changes associated with crossbridge formation and strain were studied by X-ray diffraction, electron microscopy, and mechanical methods.

A possible mechanism for force development in rigor muscle fibers subjected to lateral electrostatic expansion was discovered by electron microscopy. The thick, myosin-containing filaments were found to separate into three subfilaments in the periphery of the A-band when lattice expansion occurs. The associated decrease in axial length of the subfilaments could account for the significant axial forces that appear under these conditions.

Cryomicroscopy and image processing were used to measure the mass associated with the thick and thin myofilaments in relaxed and rigor rabbit muscle fibers. Mass transfer from the thick to the thin filament took place when relaxed fibers passed into rigor. Phases were calculated for the first five equatorial X-ray reflections in the relaxed and rigor states.

Gel electrophoresis showed that muscle fibers from Duchenne muscular dystrophy patients contain normal amounts of all the muscle proteins (including titin and nebulin). Absence of nebulin does not appear to be the cause of this disease.

Radiation inactivation analysis of force development by muscle fibers at various sarcomere lengths suggests that titin and nebulin may directly affect the force output of actomyosin crossbridges as well as order of the thick filaments in activated muscle fibers.

The overall goal of the project is to find out how the actomyosin system in muscle fibers produces force and motion, and how the mechanical responses are regulated.

Project Description:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.

2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.

3. Electron microscopy of muscle fibers fixed under various conditions of ionic strength and pH.

4. Radiation inactivation analysis of active and passive processes in muscle fibers.

Major Findings:

1. Rigor muscle fibers expand laterally and shorten when ionic strength is lowered and/or pH is raised relative to the physiological value. Electron microscopy showed that this expansion is associated with the separation of the thick, myosin-containing filaments into three subfilaments in the peripheral parts of the A-band. Shortening appears to be caused by the decrease in axial length of the subfilaments resulting from thick filament dissociation. The lateral distance between the actin filament and the myosin subfilaments was nearly independent of the myofilament lattice spacing, indicating that the rigor crossbridges are rigid laterally.

2. Cryomicroscopy followed by image processing was used to measure the distribution of mass in cross-sections of muscle fibers that were quickly frozen (without fixation) in both the relaxed and the rigor states. The mass associated with the thin, actin-containing filaments was greater in the rigor state than in the relaxed state. The phases of the first five equatorial reflections were calculated to be ++--+ in the relaxed state and ++--- in the rigor state.

3. The influence of sucrose, a commonly used cryoprotectant, on the equatorial X-ray diffraction pattern of skinned muscle fibers was directly measured. Sucrose (15%) was found to decrease the intensities of the equatorial reflections, and to change the intensity ratio, I_{10}/I_{11}, about two-fold.

4. Muscle biopsies were taken from patients with Duchenne muscular dystrophy (DMD) and individual muscle fibers were assayed for contractile force and muscle protein content. The average muscle force/unit area in DMD fiber was slightly less than control value, but protein content (including titin and nebulin) was almost the same in DMD and in normal fibers. The results suggest that the nebulin content of muscle fibers is not controlled by the DMD gene, and that the disease may be caused by a decrease in the number of muscle fibers

in a given muscle.

5. Radiation inactivation was used to study the role of the megadalton cytoskeletal proteins, titin and nebulin, in muscle fibers activated by calcium at sarcomere length 1.6 μm, where ordering of the thick filaments would not be expected to affect force development. The target size for force production was about 2 megadaltons, which suggests that titin and nebulin may directly affect the actomyosin crossbridges as well as order the thick filaments in calcium-activated muscle fibers.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of molecular mechanism of muscular contraction, together with the chemistry of the activation process, is useful in the rational handling of musculoskeletal, cardiovascular, and arthritic diseases.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, nucleotide) on the ability of muscle fibers to develop force and shorten will be examined directly in skinned fiber segments.

2. The influence of these same agents on myofilament spacing and crossbridge configuration will be examined in skinned fibers by X-ray diffraction, using both conventional and synchrotron radiation sources.

3. The effect of motion on the number of crossbridges in skinned fibers will be studied by X-ray diffraction.

4. Radiation inactivation analysis will be used to study the role of very large molecules in contractile function.

Publications:

Podolsky, R. J., and Arata, T.: Force Generating Mechanisms in Striated Muscle. In Sugi, H., and Pollack, G. H. (Eds.): Molecular Mechanism of Muscle Contraction. New York, Plenum, in press.

Horowits, R., and Podolsky, R. J.: A-band positional stability in striated muscle fibers depends on sarcomere length: Evidence for the role of titin filaments. J. Cell Biol., in press.

Reported in press, 1986:

Horowits, R., Kempner, E. S., Bisher, M. E., and Podolsky, R. J.: A physiological role for titin and nebulin skeletal muscle. Nature (Lond.) 323: 160-164, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 27001-13 LPB

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Contractility of Skeletal and Smooth Muscle

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Mark Schoenberg	Medical Officer	LPB, NIAMS
Others:	V. A. Barnett	Staff Fellow	LPB, NIAMS

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Muscle Biophysics

INSTITUTE AND LOCATION
NIAMS, NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	0

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To better understand how the force-producing myosin crossbridge interacts with actin, we have measured the crossbridge detachment rate-constants in rabbit skeletal muscle fibers and compared them with those for detachment of myosin subfragment-1 from actin in solution. We find that, in the fiber, crossbridge detachment is not described by a single rate-constant but by multiple rate-constants spanning a wide range. The fastest rate-constants are comparable to the detachment rate-constant of S1 from actin in solution but the majority are much slower. In FY '86 we showed that part, but not all, of the slowness in the apparent detachment rate-constants could be related to crossbridge clustering.

In FY87 we examined how the rate-constant for crossbridge detachment in the presence of MgPPi depended upon MgPPi concentration. It was shown that if crossbridge heads act independently, the concentration dependence of the rate of crossbridge detachment should have the same form as the Michaelis-Menten equation. However, we found experimentally that the concentration dependence relationship was sigmoidal. The most likely explanation for our finding was that both of the S1 heads of a crossbridge need to bind MgPPi before either has a high probability of relaxing a substantial fraction of the tension it supports. This, in turn, provides an additional explanation for why the crossbridge detachment rate-constants in the fiber are smaller than the corresponding rate-constants in solution. If two heads of a crossbridge need to detach simultaneously before either head can relax the tension it supports, this process would indeed be expected to be slower than the rate at which S1 detaches from actin in solution.

We also determined what fraction of time a crossbridge with bound ATP spends attached to actin in relaxed rabbit psoas fibers. For fibers that have had their sarcolemma made permeable, this number is 50-100% at very low ionic strength and 3-5% at physiological ionic strength. For intact fibers this number is estimated to be 5-10%.

OBJECTIVES:

To understand the molecular and structural basis of muscle contraction.

METHODS AND MAJOR FINDINGS:

1) Kinetics of the MATP crossbridge.

Several years previously we discovered and characterized the weakly-binding crossbridge state seen in relaxed fibers when ATP binds to the active site. That state showed rapid, reversible attachment and detachment of crossbridges, with detachment rate-constants in the neighborhood of 10^4 s^{-1}. By making the sarcolemmas of our muscle fibers permeable to the bathing medium, it was possible to control the internal milieu, including internal ionic strength, of the fibers. Our original experiments were performed at very low ionic strength in order to maximize the amount of time a weakly-binding crossbridge would spend attached to actin. An important question that remained following the initial study was how much time the crossbridges actually spend attached to actin at physiological ionic strength. In order to answer this question, in FY '87 we investigated the attachment and detachment rate-constants, and the equilibrium binding constants, of the MATP crossbridge as a function of ionic strength. Experimentally we found:

1) The crossbridge detachment rate-constant in permeable rabbit psoas fibers at 5°C is about 10^4 s^{-1} at low ionic strength and is not greatly affected by ionic strength.

2) The percentage of time an MATP crossbridge spends attached to actin decreases about 20-fold as ionic strength is increased from very low values, μ = 20 mM, to physiological values μ = 170 mM.

These findings imply that the main effect of increasing ionic strength is to cause a big decrease in the crossbridge attachment rate-constants. Since the crossbridge with ATP bound spends 50-100% of its time attached to actin at low ionic strength, the corresponding number at high ionic strength is 3-5%. The demonstration that crossbridges attach to actin at all ionic strengths suggests that, just as we showed previously at low ionic strength, muscle relaxation at normal ionic strength is caused primarily by blockage of a step subsequent to attachment, and not by blockage of the attachment step.

2) Magnitude of the fiber crossbridge detachment rate-constants relative to the detachment rate-constant of myosin subfragment-1 from actin in solution.

It has been postulated that an unstrained crossbridge should detach, in a fiber, with about the same rate-constant as myosin subfragment-1 (S1) detaches from actin in solution. In the presence of the ATP analogue magnesium pyrophosphate, muscle crossbridges detach not with a single rate-constant but with a wide range of detachment rate-constants. The fastest of these rate-constants are comparable to the detachment rate-constant of S1 from actin in solution but many of the crossbridges detach with rate-constants perhaps 100-fold slower than the S1 detachment rate-constant. In FY '86 we showed that some of the slowness in the fiber detachment rate-constants relative to

solution could be due to the fact that in the fiber the crossbridges tend to bind clustered together. However, this effect was not large enough to explain the entire phenomenon.

In FY '87 we very carefully examined how the rate-constant for crossbridge detachment in the presence of MgPPi depended upon MgPPi concentration. It was shown that if crossbridge heads act independently, the concentration dependence of the rate of crossbridge detachment should have the same form as the Michaelis-Menten equation. However, what we found experimentally was that the concentration dependence relationship was sigmoidal. It was shown that the most likely explanation for our data was that both of the S1 heads of a crossbridge need to bind MgPPi before either has a high probability of relaxing a substantial fraction of the tension it supports. This provides an additional explanation for why the crossbridge detachment rate-constants in the fiber are smaller than the corresponding rate-constants in solution. If two heads of a crossbridge did need to detach simultaneously before either head could relax the tension it supports, this process would indeed be expected to be slower than the rate at which S1 detaches from actin in solution.

3) Study of crossbridge interactions by means of sulfhydryl modification.

Two new areas of investigation were started in FY '87. One involved the use of the crosslinking agent para-phenylenedimaleimide. This agent is known to cross-link the SH_1 and SH_2 sulfhydryl groups of the crossbridge head, thereby greatly reducing the ability of the head to bind to actin. If we are able to effectively prevent some of the crossbridge heads from binding using this reagent, this should provide an excellent opportunity for examining cooperativity between heads (see item #2). We have already found that this crosslinking agent is capable of crosslinking the crossbridge head to actin and may be an excellent probe of crossbridge binding to actin under conditions where other probes are inadequate.

4) Regulation of contractile activity in smooth muscle.

Another new area of inquiry in FY '87 involves a joint collaboration with the Laboratory of Molecular Cardiology, NHLBI. We have initiated a joint biochemical-physiological study of the regulation of smooth muscle, with the Laboratory of Molecular Cardiology providing the biochemical expertise. Their reference to this project is Z 01 HL 04202-06 MC. Our initial studies have been concerned with the role of protein kinase C in activation of smooth muscle. It is known that activation of smooth muscle is largely controlled by phosphorylation of the 20,000 Dalton myosin light chains. To date we have found that:

1) Phorbol esters, believed to be activators of protein kinase C, are capable of stimulating bovine tracheal smooth muscle to contract.

2) This contraction is accompanied by phosphorylation of the 20 KDalton myosin light chain at two sites; the serine 19 site that is phosphorylated by myosin light chain kinase and a second site, probably the serine 1 site thought to be associated with protein kinase C stimulation.

Work is continuing in order to quantitate this effect and to find out whether all the light chains are phosphorylated at two sites or whether there are two populations of light chains phosphorylated at different sites. Also the state of the smooth muscle fiber after phorbol ester stimulation will be compared to that caused by catecholamine stimulation. The latter agent causes contraction via a mechanism involving phosphorylation of the myosin light chains by myosin light chain kinase.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Experiments on the mechanism of muscular contraction are important in that they contribute to a sound understanding of how normal muscle works. This, in turn, is useful in understanding diseased muscle. Furthermore, the high degree of organization of the contractile proteins in skeletal muscle makes this a particularly useful system for elucidating contractile mechanisms, some of which may be common to the cellular and subcellular motions of other, less easily studied cells.

FUTURE COURSE:

Use of sulfhydryl reacting agents has shown great promise as a probe of crossbridge function and work along these lines will be continued. Examination of the crossbridge detachment rate-constants at very high ionic strength, where only a few crossbridges are attached, should shed additional light on crossbridge cooperativity. Finally, exploration of a possible physiological role for protein kinase C stimulation will continue.

PUBLICATIONS:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 27002-09 LPB
FORMERLY
Z01 AM 27009-08 LCDB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Macromolecular Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Alasdair C. Steven, Visiting Scientist - Section Chief	LPB, NIAMS
Adelia C. Bauer, Physiologist	LPB, NIAMS
Margaret E. Bisher, Microbiologist	LPB, NIAMS

COOPERATING UNITS *(if any)*
Computer Systems Laboratory, Division Computer Research and Technology, NIH (B.L. Trus); Biology Department, Brookhaven National Laboratory, (J. Hainfeld, J. Wall); Other collaborators as specified below.

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Laboratory of Physical Biology

SECTION
Section on Structural Biology

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
03	03	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structures and mechanisms of assembly of macromolecules and marcromolecular complexes are studied, primarily by high resolution electron microscopy complemented with computer image processing. Over the past year, our major efforts have been devoted to analysis of (i) the tail-fibers of bacteriophage T7; and (ii) frozen-hydrated transverse thin sections of skeletal muscle in the relaxed and rigor states.

(i) The T7 tail-fiber represents an experimentally tractable example of a viral receptor-recognition protein. We have combined piece-wise correlation averaging of electron micrographs of molecules prepared both by negative staining and by freeze-drying without staining, with predictions based on the known sequence of the gp17 protein (553 residues), to formulate a detailed model of this fibrous protein. The fiber is a trimer and consists of three domains. The proximal and distal half-fibers are both rods ∿16nm long, joined at a kink-angle of ∿90°, and are linked to the virion by the third domain. Of particular interest is the proximal half-fiber which is a triple-stranded coiled-coil of α-helices, one of the few well-authenticated examples of this conformation.

(ii) We have developed and applied methods for the analysis of frozen-hydrated transverse thin sections of muscle. Such images combine the advantages of good preservation of native structure (unlike conventional thin sections) and phase determinacy (unlike X-ray fiber diffraction). The results reveal differences between the distributions of density in muscle in the relaxed and rigor states.

Project Description:

Objectives: To elucidate the structural organization, design principles, and functional significance of macromolecular assemblies. Specific systems under study include virus particles, cytoskeletal protein filaments, clathrin-coated membranes, and bacterial fimbriae.

Experimental Approach: This objective requires a multidisciplinary approach, integrating information from diverse experimental sources (biochemical, biophysical, ultrastructural, etc.). Our strategy is to establish the structural basis in projects which are usually collaborative in nature. In terms of specific techniques, we are engaged in:

1) high resolution electron microscopy,
2) image analysis and enhancement by optical diffraction and computer image processing,
3) development of appropriate specimen preparation techniques for electron microscopy,
4) computer-based analysis of amino-acid sequences and gel electrophoregrams.

Statement of Progress:

A. Development of Experimental Facilities:

1. Development of Computer Image Processing Resources (in collaboration with Dr. B.L. Trus, CSL/DCRT and Dr. M. Unser, BEIB). Software development has continued as specific needs have arisen, within the overall strategy of maintaining an integrated system of mutually compatible programs (PIC). During the past year, we have extended our capacity to analyze sets of images of single particles, by deriving an optimized measure of resolution that is applicable to such data sets (assuming translational and orientational alignment to have been achieved). This measure is consistent with the more restrictive resolution criterion for crystalline specimens defined by the extent of periodic reflections in their diffraction patterns.

In order to expand our image processing capabilities, a facility based on a Microvax II has been planned (with Mr. R. Fico and Mr. T. Kuhfuss, CSL/DCRT) and is currently under procurement. This facility will be implemented in our laboratory, and will ultimately be linked to the Image Processing Facility in DCRT (which we have used hitherto) by means of a video- rate network.

2. Scanning Transmission Electron Microscopy. We have continued to make extensive use of the Scanning Transmission Electron Microscope, which operates as an NIH Bio-technology Resource at Brookhaven National Laboratory (collaboration with Drs. J. Wall and J. Hainfeld, BNL). The current *modus operandi* is to convey specimens from NIH to Brookhaven for observation: the resulting images are then brought back to NIH for quantitation, processing, and related analyses.

B. RESEARCH PROGRAM: (Summary of Progress and Proposed Future Course)

B-1 THE STRUCTURE OF VIRUSES AND VIRUS-RELATED PARTICLES:

(a) High-Resolution Structure of the GP17 Tail Fibers of Bacteriophage T7 (collaboration with Drs. J.V. Maizel, LMB/NCI; B.L. Trus CSL/DCRT; F.W. Studier, Brookhaven National Laboratory).

Six filamentous appendages composed of the protein gp17 (61 kDa) are attached to the conical tail tube of phage T7. Their presence is necessary to give an infectious virus particle, and they are thought to act by recognizing specific receptors on the surface of a susceptible bacterium, subsequently triggering injection of DNA to initiate infection. We have now refined our molecular model of the fiber, which is envisaged to be a gp17 trimer, with the three polypeptide chains assuming extended conformations in parallel association. The amino-terminal domains (residues 1-150) link the fiber to the tail-tube. Residues 151-267 form a triple-stranded α-helical coiled coil, arguably the best-documented example to date of this somewhat unusual conformation (vis-a-vis the relatively common double-stranded coiled-coil). Residues 268-553 form the alignment of four globular nodules in the distal half-fiber. We infer that in the coiled-coil of the proximal half-fiber, the three chains may not be in strict axial register, and they are distinctly non-equivalent in the immediate vicinity of the kinked joint between the two half-fibers. We have identified a short homology between a carboxy-terminal tetrapeptide and the corresponding sequence in the tail-fiber of phage T4. In view of the similarity between their respective host ranges (specified by the bacterial lipopolysaccharide), this sequence may represent (at least part of) the receptor-combining site. Continuing experiments are intended to test this hypothesis and to give a more detailed specification of molecular folding in the distal half-fiber.

(b) Assembly-dependent Conformational Changes of the gp23 Surface Lattice of Bacteriophage T4D Capsid. (A. Bauer; collaboration with Dr. L.W. Black, University of Maryland Medical School, Baltimore). During the course of T4 head assembly, gp23, its major capsid protein, assumes a specifically regulated sequence of conformational states. Conformational changes between successive states are irreversible, they are effected *in situ* on the polymerized protein, and they involve large-scale structural changes (such as a 2.0nm increase in the hexagonal lattice constant). We are continuing to investigate the structural basis of these remarkable conformational transtions. Specifically, we have applied computational algorithms to the sequence of gp23 to detect potential antigenic sites by the criterion of local hydrophilicity. For the six most promising sequences, synthetic peptides of 8-10 residues each have been prepared (collaboration with Dr. F. Robey, BB/FDA), and used to generate polyclonal antibodies to map (by immuno-electron microscopy) the corresponding peptides in each conformational state of the surface lattice, i.e. is a particular determinant surface-exposed and immunologically recognisable in a given state? is it located on the inner or the outer surface? where is it located in the plane of the hexagonal unit cell?

Of the six peptides synthesized, one has proved to be strongly antigenic, one moderately antigenic, two slightly antigenic, and the remaining two essentially

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non-antigenic by the criterion of ELISA assay with the appropriate peptide. The strongly reactive antigenic peptide resides in the so-called delta-region of gp23 i.e. the amino-terminal 65 amino-acids that are proteolytically removed in the course of capsid maturation. Antibodies against this peptide react strongly with the inner surface of "native polyheads", i.e. tubular polymers which are polymorphic variants of the precursor capsid. A similar reaction is evinced by affinity-purified antibodies directed against the cleavage site (one of the mildly antigenic peptides). Current work on this system is aimed at completing the project by characterizing the reactivity of the remaining two antisera.

Experiments have also been designed to characterize the effects on the structure and stability of native polyheads of incubating them at 50°C. This treatment is thought on the basis of earlier calorimetric studies (P.Ross, L. BLack, M.E. Bisher and A.C. Steven, J. Mol. Biol. 183: 353-364, 1985) to result in thermal denaturation of the delta region while leaving the rest of the gp23 surface lattice unaffected.

(c) The Intramural AIDS Targeted Antiviral Program. A multidisciplinary task-force has been mobilized within the NIH intramural research community to bring the experimental methods of modern biology to bear on the molecular constituents of the Human Immunodeficiency Virus (HIV), the causative agent of AIDS. We are participating in this program with the aim of applying to gp160 and gp120, the surface glycoproteins of HIV, the methods of structural analysis which we have developed in our work on the corresponding protein of phage T7 (cf. (a) above). A structural map of HIV proteins, even at relatively modest resolution, may be of use in vaccine design, and perhaps also as an aid in early stages of high resolution structure determination by X-ray crystallography.

B-2 INTRACELLULAR PROTEIN FILAMENTS: THE CYTOSKELETON

(a) Structure of Intermediate Filaments (IF) (collaboration with Dr. P.M. Steinert, DB/NCI; Dr. B.L. Trus, CSL/DCRT). These polymers constitute one of the three major filament systems in the cytoskeletons of eukaryotic cells, along with microfilaments and microtubules. IF are the only such system whose structure(s) is not yet known at the level of subunit packing. Moreover, they are the only system which exhibits pronounced variability with respect to its constituent protein subunits.

In the past year, we have prepared and published a report on the main lines of new evidence that emerged at the Fogarty International Symposium on Intermediate Filaments which we organized at NIH in September, 1986. Our current experimental activity on IF is two-fold: to map the hypervariable end-domains by electron microscopic methods, and to deduce likely conformational features of end-domain sequences from predictive computer analysis of end-domain sequences. In this respect, the quasi-repetitive Gly/Ser-rich sequences characteristic of the end-domains of some epidermal keratins have been found to have certain properties suggestive of the "omega-loop", a secondary structure recently recognized to occur commonly in the surface loops of many globular proteins (Leszczynsky & Rose, Science 234: 849-855, 1986).

(b) Different Physiological States of Vertebrate Skeletal Muscle Visualized In Frozen-Hydrated Thin Sections. (collaboration with Dr. B.L. Trus, CSL/DCRT; Dr. R.J. Podolsky, LPB/NIAMS; Drs. A. McDowall and J. Dubochet, EMBL, Heidelberg, F.R.G.). Conventional approaches to visualization of the force-generating interactions between actin and myosin filaments in the myofilament lattice have been based on x-ray fiber diffraction and thin section electron microscopy. However both techniques have their limitations. The x-ray diffraction experiments provide sets of Fourier amplitudes from "physiological" material, but lack the phase information required to completely specify the corresponding electron density maps. With conventional thin sectioning methods, the "nativeness" of the muscle structure thus represented is suspect. The recently developed technique of cryo-electron microscopy has the potential for direct imaging of preparations maintained in their native state.

We have devised image processing methods to compensate for the adverse effects of non-linear contrast transfer (incurred by the de-focussed bright-field imaging used in this type of electron microscopy), and for the various kinds of spatial disordering to which frozen-hydrated sections are particularly susceptible. These methods have been applied to sets of micrographs obtained by our collaborators at EMBL, Heidelberg, and this phase of the work is almost finished.

This project has been strengthened over the past year by the arrival of Dr. Maria Barbosa, an NRC Fellow associated jointly with the SSB and the SMB/LPB. Dr. Barbosa is implementing the experimental equipment required to obtain cryo-section micrographs in this laboratory. In addition, she made several sets of conventional thin sections of rabbit muscle in rigor and relaxed states under carefully standardized conditions, to provide the basis for a quantitative comparison between the cryo-section and the conventional thin section representations of the myofilament lattice in axial projection.

B-3 BIOMEDICAL APPLICATIONS OF ELECTRON MICROSCOPY.

(a) Physical Basis of Protein Coloration in Silver-Stained Polyacrylamide Gels. (collaboration with Drs. C. Merril and M. Harrington, CNB/NIMH). Under favorable circumstances, silver-staining can increase the detection sensitivity of electrophoretically separated proteins by a factor of 50-100 over that achieved with Coomassie Blue, the most commonly used organic stain. Whereas most proteins exhibit a brownish color upon silver staining, numerous observations have been made of proteins that exhibit other colors. Colorability is a property with considerable analytical potential in that it may eventually become possible to deduce chemical properties of a protein on the basis of its propensity for coloration when subjected to specific silver-staining protocols. In this context, we have attempted to establish the physical basis of color effects by testing the alternative hypotheses (a) that colors are caused primarily by the growth of microscopic silver grains of particular sizes, or (b) they are due to conjugate bond systems created by the formation of specific molecular complexes between a protein and one (or a few) silver atoms. For this purpose, we have performed thin sectioning electron microscopy of colored bands excised from silver-stained polyacrylamide gels. The results strongly favor hypothesis (a) in the sense that silver grains in a yellow-staining band are typically much smaller

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(∿40nm in diameter) and more homogeneous than those from a blue-staining band (grain diameters of 40-110nm). Thus the mechanism of protein coloration in gels appears to be broadly analagous to that of silver-based color photography.

Significance to Biomedical Research and the Program of the Institute:

An improved understanding of macromolecular structures and the mechanisms that control their assembly - particularly in such contexts as regulation of the cytoskeleton, viral propagation, and membrane biogenesis - is of direct relevance to many areas of biomedical research. The long-term aim of work on virus assembly, in both model and clinically significant systems, is to be able to control (and where necessary to suppress) the multiplication of viruses during the course of viral infections. In addition to helping to elucidate the basic biology of these phenomena, a further goal of structural work is the identification of sub-cellular and supra-molecular symptoms of pathological conditions.

Publications:

Fraser, R.D.B., Steinert, P.M. and Steven, A.C.: Focus on Intermediate Filaments: Trends in Biochem. Sci., 12: 43-45, 1987.

Cowell, J.L., Zhang, J.M., Urisu, A., Suzuki, A., Steven, A.C., Liu, T., Liu, T.-Y and Manclark, C.R.: Purification and Characterization of Serotype 6 Fimbriae from Bordetella pertussis and Comparison of their Properties with Serotype 2 Fimbriae: Inf. Immun., 55: 916-922, 1987.

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Steven, A.C.: Intermediate Filament Structure - Diversity, Polymorphism, and Analogy to Myosin. In Goldman, R.D. and Steinert, P.M. (Eds.): Intermediate Filaments. New York, Plenum Press 1987 - in the press.

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Horowits, R., Kempner, E.S., Bisher, M.E. and Podolsky, R.J.: A Physiological Role for Titin and Nebulin in Skeletal Muscle: Nature (Lond.), 323: 160-164, 1986.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 27003-28 LPB
FORMERLY
Z01 DK 23860-27 LBP

PERIOD COVERED
March 15, 1987 through September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biophysical Studies of Metabolic Activity and Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Ellis S. Kempner, Ph.D., Physicist and Chief,
Section on Macromolecular Biophysics LPB NIAMS

COOPERATING UNITS *(if any)*
Drs. M.J. McCreery (Letterman Army Institute of Research); S. Pestka (Roche Institute); R. Wood (University of Georgia); R. Salovey (University of Southern California)

LAB/BRANCH
Laboratory of Physical Biology

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Section on Macromolecular Biophysics

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NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project was transferred from the Section on Physical Biology, Laboratory of Biochemical Pharmacology, NIDDK, on March 15, 1987

Target analysis of radiation inactivation had three aspects:

1. Theoretical studies developed a target analysis for complex systems which show a radiation-induced change inintrinsic activity (Km for enzymes, KD for receptors).

2. Fundamental studies of radiation action which showed the same temperature effect previously found for enzymes also holds for synthetic polymers, and found that the phenomenon is due to the temperature-dependence of free radical generation. Other fundamental studies proved that radiation action on the oligosaccharide portion of glycoproteins did not contribute to the target size, and this was due to the absorption of the radiation energy near the primary ionization.

3. The method was applied to several new biochemical systems, and most notably to a complex physiological system, muscle contraction.

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Project Description:

Radiation Inactivation and Target Analysis for Determination of Molecular Size

Continuing studies of the method of radiation inactivation have focussed on three areas: theoretical development of radiation destruction of complex systems; experiments of test systems to examine fundamental questions about radiation damage; and application of the technique to a wider range of biological processes.

Theoretical studies concerned enzymes and receptors which show unusual radiation responses: changes in Km or KD respectively. If the radiation dependence of the change can be established, these effects can be incorporated into the classical target analysis. This extension of target theory permits calculation of the molecular size of complex functional units, such as the mouse brain nitrendipine receptor.

We previously showed that the radiation sensitivity of enzymes was dependent on the temperature during exposure, and the effect was quantitatively the same in all enzymes. This has now been extended to synthetic polymers. The same temperature dependence occurs in many chemical reactions occurring in irradiated plastics. The one notable exception was the radiation-induced formation of transvinylene, which is independent of temperature. The mechanism of this process is a direct action of radiation at the site and does not involve free radicals which are common to most other radiation mechanisms. Based on previous radiation studies of hexadecane, it is likely that the fundamental temperature-dependent process is the radiation production of the free radical. Additional temperature dependencies also may arise in specific chemical reactions involving these free radicals but all free radical radiation effects will display at least the one temperature effect in common to all macromolecules.

This laboratory previously showed that the target size for the glycoprotein invertase corresponded to the protein moiety only; the covalently-attached oligosaccharide did not contribute to the radiation-determined molecular size. Using a synthetic glycoprotein (glucose 6-phosphate dehydrogenase coupled to agarose), it was shown that the effect was independent of the protein or the nature of the carbohydrate linkage. The target sizes of fifteen natural glycoproteins have been determined (eleven in this laboratory), and all are now seen to be independent of attached carbohydrate. In pure oligosaccharides, radiation damage is confined to a small region of the molecule; thus in a glycoprotein radiation energy deposited in the carbohydrate should be absorbed locally and would not be transferred to the protein chain.

The radiation technique was applied this year to several important biological systems, including cholyl CoA synthetase and glutathione S-transferase. The most unique application was to the study of muscle contraction (covered elsewhere in this report); for the first time, target analysis was successfully used *in situ* on a complex multistep biological function and revealed unique molecular events.

Publications

Horowits, R., Kempner, E.S., Bisher, M.E. and Podolsky, R.J.: A physiological role for titin an nebulin in skeletal muscle. Nature 323: 160-164, (1986)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 27004-18 LPB
Formerly
Z01 DK 23600-17 LBP

PERIOD COVERED
March 15, 1987 through September 30, 1987

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
The Dynamic Properties of Cell Membranes and Related Systems

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI: Norman L. Gershfeld, Ph.D. Research Chemist LPB NIAMS

COOPERATING UNITS (*if any*)
Dr. Ralph J. Nossal. PSL, DCRT, and Dr. Stanley I. Rapoport, LN, NIAMS

LAB/BRANCH
Laboratory of Physical Biology

SECTION
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- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The principal goal of this project is to understand the molecular mechanisms utilized by cells for assembling the membrane lipid bilayer. From studies of aqueous equilibrium dispersions of a wide variety of synthetic phospolipids a model for membrane bilayer assembly has been developed which asserts that the membrane bilayer is unilamellar and spontaneously assembles _in situ_ at a critical temperature T*, (the growth temperature), with a composition that is determined by lipids in the metabolic pool of the cell. From studies of the total lipid extracts from erythrocytes, bacteria, and rat cortex, cerebellum and brain stem the model has been shown to be generally applicable to a wide range of cell types and organisms. A corollary of this model for assembly predicts that when temperatures are above or below T* the unilamellar state will transform to multibilayers. For mammalian cells which can synthesize lipid only at body temperatures, it is predicted that when temperatures are raised regions of the membrane will become lipid-deficient because of the unilamellar-multibilayer transformation, and become leaky perhaps leading to cell death. From kinetic studies of erythrocytes and lipid dispersions the transformations have been shown to be very slow processes. It is believed that the slowness of this transformation permits cells to withstand temperature fluctuations around T*. The critical unilamellar state model helps explain why erythrocytes hemolyze under the mild conditions of pyrexia where hemolysis has been observed even with a one degree elevation of body temperature. The instability aspect of this critical bilayer model is currently being tested as a possible basis for membrane failure in Alzheimer's disease.

Mechanisms of Cell Membrane Assembly and Disassembly

The present objective of this project is to establish the mechanisms utilized by cells for assembly of their membranes. Our studies have focussed on processes which form membrane lipid bilayers, and in particular on the conditions which lead to the assembly of the unilamellar or single bilayer state. More recent studies indicate that membrane lipids assemble spontaneously in situ into the unilamellar state at the growth temperature of the cell; the composition of the membrane is determined by the lipids that are present in the metabolic pool of the cell. Furthermore, it has been deduced that the growth temperature is a critical point T* at which the unilamellar state is optimally stable. At temperatures above and below T* non-unilamellar lipid structures form. This result was deduced from thermodynamic properties of aqueous dispersions of the total lipid extracts from various cell membranes, and of pure synthetic phospholipids. These deductions have been supplemented with more direct quasi-elastic light scattering studies in which unilamellar (single bilayer) vesicle formation has been observed at the critical temperature T* in dispersions of a typical phospholipid dimyristoylphosphatidylglycerol. Unilamellar vesicles were observed only at T*; at temperatures above and below T* multibilayer structures were observed.

Implicit to the concept that the growth temperature is a critical point is the corollary that the stability of the membrane bilayer will be exquisitely sensitive to minor alterations in temperature and composition. According to this view of the membrane structure, if the ambient temperature of the cell deviates from T* the membrane must shift from the normal unilamellar to a multibilayer state. For cells with a fixed surface area this model predicts that regions of the membrane will be deficient in lipid, and perhaps be accompanied by traumatic disruptions of membrane function. Since most cell preparations appear to be viable over a relatively wide temperature range. This model for membrane disassembly raises an important issue concerning cell stability. This question has now been examined in several systems and a partial understanding of how membrane bilayers respond to thermal fluctuations has been reached. For poikilotherms, such as bacteria, it has been shown that the cells accomodate to growth temperature shifts by synthesizing new membrane lipid whose composition conforms to the assembly requirements of the new T*. However, for most homeotherms which cannot accomodate to new ambient temperatures by synthesizing new lipid, the cells become leaky when temperatures are elevated above T*. Just how crucial this property of the critical bilayer state is to the viability of mammalian cells has been examined in red blood cells. From kinetic studies of thermally induced hemolysis it has been demonstrated that an elevation of even one degree above normal body temperature is sufficient to cause hemolysis. The hemolysis does not involve membrane protein because independent studies indicate that all erythrocyte enzymes are functioning at nearly optimal rates, and spectrin structure is normal. We have shown however, that the proposed unilamellar-multibilayer transformation for temperatures exceeding T* conforms to the observed energetics of hemolysis. It appears that red blood cells can withstand thermal flucteratious because of the slow rate of the unilamellar-multibilayer transformation.

The critical bilayer state also predicts that the bilayer would become unstable when ambient temperatures are below T*. However, we have demonstrated with dispersions of phospholipids that the critical unilamellar state supercools when temperatures are lowered from T*.

The concept of the critical bilayer state is currently being examined in the context of possibly providing an explanation for membrane failure in diseased states. Under study at present are lipid extracts from the cortex and cerebellum of Alzheimer patients. Lesions are generally localized to the cortex. In preparation for these studies, control experiments have tested whether lipids from a tissue as complex as brain exhibit a critical temperature. Preliminary studies with lipid extracts of rat cerebellum, cortex and brain stem indicate a critical temperature for the formation of the unilamellar state in each of these tissues is 36 ± 1 °C, rat body temperature.

Publications

Gershfeld, N.L. Phospholipid surface bilayers at the air-water interface. III. Relation between surface bilayer formation and lipid bilayer assembly in cell membranes. Biophys. J., (1986) 50: 457-461.

Gershfeld, N.L., Stevens, W.F., Jr., and Nossal. R.J. Equilibrium studies of phospholipid bilayer assembly: coexistence of surface bilayers and unilamellar vesicles. Faraday Discussions Chem. Soc. (1986) 81: 19-28.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AR 27005-5 LPB FORMERLY Z01 DK 15405-4 LCDB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Aggregation of Human Platelets Induced by Decommpression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Makio Murayama, Ph.D. Research Chemist, LPB, NIAMS

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SECTION
Section on Macromolecular Biophysics

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NIAMS, NIH, Bethesda, MD

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 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We previously reported that human platelets aggregate spontaneously when decompressed to 1/3 atmosphere (Thrombosis Res. 33: 477, 1984) and that fibrinogen-dependent DIPA (decompression-inducible platelet aggregation) is inhibited by 10mM menthol, 10mM menthone, or 5 μM thymol (Thrombosis Res. 42:511, 1986). It is suggested that oppositely charged amino acid residues of fibrinogen are juxtaposed in the specific binding sites(s). Under compression, electrically charged groups gain layers of water molecules which are pulled in closer to the center of the charge, making the layer more compact. This increases the thickness of the shield, disaggregating the platelets. Conversely, decompression allows the water to diffuse away from the compact shield; water molecules are randomized into the bulk phase; the shield becomes thinner, permitting spontaneous aggregation of platelets. Increasing the concentration of neutral salts inhibits platelet aggregation because high ionic strength attenuates the electric charge and thickens the shield. Molecules having a large electric dipole moment inhibit platelet aggregation by a similar mechanism. The theory has been experimentally confirmed (Thrombosis Res. 45; 729, 1987). Electric constriction appears to be involved in fibrin(ogen) dependent DIPA. The tetrapeptide R.G.D.S. (Arg-Gly-Asp-Ser) is found in both the specific binding site on the membrane surface of platelets as well as on the alpha-chain of fibrinogen. Synthetic tetrapeptide R.G.D.S inibits DIPA; 3 μM solution R.G.D.S changes its electric conductivity as a function of pressure, indicating dissociation under compression and association under decompression. The juxtaposed amino acid residues may be positively charged Arg and negatively charged Asp. Two alpha-helices were constructed from atom models, showing the non-convalent interactions between juxtaposed and oppositely charged amino acid residues consist of hydrogen-bonding with electrostatic interaction superimposed. This finding differs from the commonly held notion that fibrin polymerization takes place by way of H-bonding and hydrophobic interaction. There is no experimental indication of any hydrophobic interactions in DIPA and fibrin polymerization.

Project Description:

Objectives: The main objective is to investigate the molecular mechanism underlying aggregation of human platelets induced by pressure changes (DIPA, acronym for decompression-inducible platelet aggregation). Also to investigate molecular mechanisms to prevent and/or inhibit DIPA. Because DIPA is a fibrinogen-dependent mechanism (as was found in our laboratory), any mechanism which will block fibrin(ogen) aggregation could also block the initial stages of human platelet aggregation by neutral salts: NaBr, sodium sulfate and potassium ferricyanide which increase the ionic strength; other reagents which will block electrostatic interactions are molecules having a large electric dipole moment such as the drug piracetam (2-oxo-pyrrolidine-acetamide) which has been found in our laboratory to block fibrin polymerization and also to inhibit DIPA. More recently, the amino acid residues involved in the specific recognition site on the membrane of platelets and in the binding site of fibrin(ogen) are believed to be the peptide segment having the amino acid sequence: Arg. Gly.Asp. Ser (R.G.D.S). The synthetic peptide causes 50% inhibition at about 3 mM. Scale model building experiments using precision atom models suggests that R.G.D.S is a sufficient and adequate sequence for juxtaposed counter-charged amino acid residues to interact in DIPA. Therefore, it seems reasonable to assume that under pressure the dimer of R.G.D.S would dissociate thus the volume of the solution would decrease due to electric constriction and vice versa. It appears that volume change takes place by way of a second mechanism: hydrogen bonding.

Major Findings

During the report period, we confirmed last year's findings; we add here our new findings. The notion that decompression alone can induce aggregation of human platelets is a novel idea; corollary to this theory is that compression can reverse and/or inhibit platelet aggregation. Platelet aggregation is the very first step in the cascade of reactions in hemostasis; that reduced pressure alone could induce platelet aggregation has been theoretically derived and demonstrated experimentally for the first time in our laboratory. The theory of DIPA was derived from the theory of absolute reaction rates (Eyring) that the molecular volume of activation (V*) of fibrin polymerization is a positive number. It was calculated from

$$\frac{d \ln k'}{d\ P} = \frac{V^*}{RT}$$

The equation states that the derivative of the log of rate constant with respect to pressure equals the molecular volume of activation (V*) divided by RT. As noted above, the molecular volume of activation is +70 cc per mole for fibrin polymerization and it is also known the fibrin(ogen) is essential for platelet aggregation. Therefore it was surmised that platelets would aggregate by decompression and also that the rate of fibrin polymerization would be accelerated. Both of these notions were experimentally confirmed.

The corollary to the theory is that high pressure (compression) could induce disaggregation and/or block aggregation. The corollary has been experimentally confirmed: platelets were compressed by a hydraulic press 690 atm. for 30 minutes, then fixed *in situ*. No aggregation was found. Thus it may be stated that high hydraulic pressure inhibits and/or reverses platelet

aggregation. High hydraulic pressure can reverse both fibrin polymerization as well as DIPA.

Methods Employed: Platelet aggregation was investigated by two ways: microscopic and the change in light absorbance with time. For the morphometric study of platelet aggregation, small Y tubes were used, fabricated from 7 mm glass tubing, as previously described. 200 microliters of human platelet rich plasma (PRP) and the substance being tested for inhibitory action were placed in one arm of the Y tube and 200 microliters of 2% (v/v) glutaraldehyde in PBS was placed in the other arm. The tubes were connected to a manifold and decompressed for 3 hours. Platelets were then fixed while still under decompression by inverting the Y tube, a wet preparation was made on a slide before the plasma protein jelled, and the specimen was examined with the DIC optical microscope.

Platelet aggregation was also investigated using the conventional nephelometric method of Born. The substance being tested and 450 microliters of PRP were placed in a siliconized cuvette and stirred at 1000 rpm at 37°C in a Chronolog aggregometer. The aggregation reaction was evaluated by changes in light transmission recorded on a 10 mV recorder trace. A baseline value was determined over a period of 90 sec., the aggregating substance (ADP), collagen or PAF wad added, and the response followed for 180 sec. This is essentially the same method as previously described. Further data were obtained at the U.S. Naval Med. Res. Inst. where the Chronolog Aggregometer was available.

Electric conductance of R.G.D.S (4 uM) solution measurement as a function of pressure. A Shedlovsky conductivity cell was connected to a specially fabricated cap for the HPV permitting an electrical connection to the Radiometer conductivity meter, Type CDM2e. The signal was fed into LKB model 2210 20 mV recorder.

High pressure vessel (HPV) for compression of platelets in situ at 680 ATM and two-compartment cylinder used for the fixation of platelets in situ under high hydraulic pressure have been previously described.

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Significance to Biomedical Research and the Program of the Institute:

By and large most cells are aggregated in their normal state. Investigation into the spontaneous aggregation of platelets appears to be important in understanding how some of the other cells are aggregated. Platelets are cytoplasmic fragments of megakaryocytes. The only known physiological function of platelets is to form the hemostatic plug. While normally helpful to survival, there are other events in which there is spontaneous aggregation of platelets which appear not to be desirable: for example, platelet aggregation induced by reduced barometric pressure of acute mountain sickness (AMS). Another instance is platelet aggregation induced by decompression in the course of surfacing after deep sea diving; here the problem is further complicated by the "bends"; understanding the basic mechanism of DIPA should be of value to find ways and means to prevent aggregation of platelets, as we have done in our laboratory. In addition, the mechanism of recompression of divers who suffer acute decompression sickness after deep sea diving can be investigated by in vitro methods; we demonstrated that compression can reverse and/or inhibit platelet aggregation. The cardiovascular problems appear to arise

from DIPA due the hydrodynamic decompression of Bernoulli effect. Hydrodynamic decompression takes place because of the paradoxical Bernoulli principle; i.e., where the velocity of the blood is high, as in arteriostenosis, pressure drops, thus the decompression. In addition, there is contact activation of platelets. Relatively low hydrodynamic decompression, superimposed upon contact activation can occur concomitantly at a bifurcation of an artery, and cause massive platelet aggregation (e.g., in a massive heart attack). We will initially investigate the molecular mechanism of DIPA and then we shall find ways and means to prevent undesirable platelet aggregation.

Proposed Course of Project:

Electric constriction appears to be an underlying mechanism of DIPA; the electrically charged amino acid residues appear to be Arginyl (plus) and Aspartyl (minus). These could interact in electrostatic interaction and also hydrogen bonding could be superimposed; a kind of hybrid bond. The H-bonding here may be analogous to water-to-ice transformation. Ice is sensitive to pressure because it is more voluminous, therefore less dense. It floats. It melts under pressure. Ice melts with an increase of ionic strength. Fibrin polymerization (and blood coagulation) could be inhibited by sodium bromide, sodium sulfate and potassium ferricanide. The adhesive site on the mebrane of platelet is glycoprotein designated IIb/IIIa, which is known to have the sequence R.G.D.S. The same amino acid sequence is found in each of the alpha chains of fibrinogen. The guanidinium residue of Arg is positively charged and the carboxyl group of Aspartyl is negatively charged. When these counter charges are allowed to interact, electric constriction is removed and the molecular volume increases (as does in water ice transformation). Like ice, aggregated platelets "melt" under high pressure, and conversely. Observations we reported can be explained by the principle of Le Chatelier.

Because the synthetic tetrapetide R.G.D.S obtained commercially was only 66 percent pure, further purification is desirable. We shall use HPLC with acetonitrile, which will sublimate completely in the final (lyophilization) step.

Other experimental methods will be refined to find out whether or not the theory proposed for the molecular mechanism of DIPA is essentially correct. To this end, the high hydraulic pressure experiment will be extended with a titanium optical cell which is thermostated, and data on the absorbance change will be recorded on Cary 17 spectrophotometer.

The competitive inhibition of DIPA by synthetic tetrapeptide R.G.D.S will be repeated by using synthetic tetrapeptide of another sort, tetraglycine (G.G.G.G) which will be the true "backbone" for the tetrapeptide. This will make an excellent control permitting more precise interpretation of the data for both the competitive inhibtion of DIPA and electric conductance change as a function of pressure.

Publication

Murayama, M.: Compression inhibits aggregation of human platelets under high hydraulic pressure. Thrombosis Res.,(1987) 45:729-739.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AR 27012-03 LPB

PERIOD COVERED October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural and Mechanical Properties of Skeletal Muscle Fibers

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Leepo C. Yu, Research Physicist, Laboratory of Physical Biology, NIAMS

COOPERATING UNITS *(if any)*

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  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of the present project is to investigate the structural aspect force generation in skeletal muscle by studying X-ray diffraction patterns from single muscle fibers under a wide range of conditions. A central question is what structural changes occur among the crossbridges (myosin) attached to actin associated with force generation. In previous years we have investigated the mass distribution of the attached crossbridges in relaxed and rigor states. Last year we obtained equatorial diffraction patterns from fully activated muscle cells at low ionic strength by using the synchrotron X-ray source in Hamburg, FRG. By comparing diffraction patterns from fibers in the relaxed, and the fully Ca++ activated, and the rigor states in the same preparation, structural changes involved in force generation may be determined.

Preliminary analysis of the results shows that upon activation the intensity of the first reflection on the equator (I10) decreased by 15% while the second reflection remains unchanged to within experimental error. Assuming that attached crossbridges in relaxed fibers are in a state prior to force generation in the crossbridge cycle, the present results show that the force generating process, the "power stroke", involves a configurational change sufficiently large that it is detectable by equatorial X-ray diffraction at 228Å resolution.

Interpretation of electron density maps of the muscle cells based on the X-ray diffraction data is complicated by the lack of phase information and the effects of limited resolution. To ensure proper interpretation, systematic model calculations were carried out to determine correlations between mass distributions on one hand, and phases and amplitudes on the other. One of the major findings is that the radial position of the center of mass of the myosin heads has strong effects on the phases and amplitudes of the reflections on the equator.

Project Description:

To study structural aspect of force generation mechanism in skeletal muscle by correlating in vivo and in vitro mechanical, biochemical and structural properties of the actomyosin interactions.

Methods Employed:

X-ray diffraction is used to probe structure of muscle at molecular level. Force, velocity, etc. generated by the muscle are determined by mechanical measurements in parallel or simultaneously with the X-ray measurements. Preparation used is single, skinned rabbit psoas muscle fibers. Such a preparation allows direct intervention of the contractile proteins, and hence direct correlation with biochemical studies is possible.

Equatorial X-ray diffraction patterns from the single fibers have been recorded in the laboratory at LPB. Recently we have carried out experiments at the synchrotron radiation facility at Deutsches Elektronensynchrotron (DESY), Hamburg, FRG. The latter generates an X-ray beam as much as 1000 times more intense than the laboratory source; thereby, many experiments involving fully activated single fibers are now feasible.

Intensities of the reflections are used to reconstruct electron density maps of muscle cells with phases chosen according to predetermined selection criteria. Since diffraction patterns can be recorded from muscle during its various activities, one is able to determine changes in mass distribution, attributable to crossbridges, in transition from one state to another.

Mechanical measurements are conducted by Dr. Bernhard Brenner.

Major Findings:

One of the central questions in understandiing force generation is what structural changes are involved during the cyclic interactions of myosin (crossbridges) with actin. Although it has long been known from X-ray diffraction that there are distinct structural changes upon activation in an intact frog muscle (e.g. Huxley & Brown, JMB, 1967), those changes are mostly due to the attachment of crossbridges, since in this preparation most of the crossbridges are detached in the relaxed state. The question remaining is what structural changes occur among the attached states associated with force generation. The purpose of the present study is to compare the structures of the crossbridges in the relaxed, the fully Ca++ activated, and the rigor states in the same preparation and under low ionic strength conditions where large fractions of crossbridges are attached in all three states, so that structural changes involved in force generation may be determined.

Equatorial X-ray diffraction patterns were obtained from single, skinned rabbit psoas fibers at an ionic strength of 50mM. To maintain the integrity of the fibers, experiments were carried by using the intense synchrotron X-ray source at DESY, Hamburg, FRG. Upon activation, the intensity of the first reflection, I_{10}, decreases by 15% with that of the second reflection, I_{11},

remaining unchanged to within experimental error. This greater change in I_{10} could not simply be due to a change in the number of attached crossbridges in the relaxed configuration, since this would cause a greater change in I_{11} rather than in I_{10} (Brenner, et al., 1984). Based on model calculations (see below), the greater change in I_{10} probably represents a radial movement of myosin mass away from the thick filament backbone, but no significant increase in the mass surrounding the thin filament. Assuming that attached crossbridges in relaxed fibers are in a state prior to the power stroke in the crossbridge cycle, the present results suggest that there is a configurational change associated with the power stroke sufficiently large to be detectable by equatorial X-ray diffraction at 228Å resolution.

In the last few years we have obtained equatorial diffraction patterns with as many as 12 reflections from single fibers under relaxed and rigor conditions. With increasing number of reflections, greater details of mass distribution could be resolved. However, reconstruction of density maps is complicated by the lack of the phase information and the effect of series termination because of limited number of reflections. To assist in proper interpretations of the reconstructed density maps, models were constructed and their diffraction patterns were calculated. Factors that affected the amplitudes and phases were systematically studied. One of the major conclusions is that the amplitudes and phases of the third and the fourth reflections ([2,0] and [2,1]) are highly sensitive to the positions of the myosin heads surrounding the backbone. Phases could change signs and amplitudes cross zero when the myosin heads move radially outward away from the backbone. Meanwhile, the amplitude of [1,0], one of the strongest reflections on the equator, is also strongly affected by the position of the myosin heads. It decrases by as much as 15 fold as the center of mass of myosin heads moves away from the backbone by 8nm. On the other hand, intensity of the second reflection, I_{11}, is not affected to the same degree.

Significance of Findings:

According to the crossbridge theory of A. F. Huxley (1957), force is generated by the cyclic attachment/detachment of crossbridges (myosin heads) to actin. There has been strong evidence for the attachment and detachment of crossbridges, but efforts have long been devoted to find evidence of conformational changes after the crossbridges attach to actin. Evidence provided by the present study improves our understanding of mechanismn of muscle contraction.

Future Plans:

We shall continue in studying the structural changes involved in force generation. First, we will obtain data at various higher ionic strengths. Secondly, high resolution diffraction patterns from Ca++ activated fibers will be studied such that greater details of mass redistribution upon activation could be determined.

Phases associated with equatorial reflections will be studied with various methods, including that of swelling. The results will correlate with model calculations.

Publications:

Brenner, B., Yu, L.C., Greene, L.E., Eisenberg, E., and Schoenberg, M.: Ca^{2+}-sensitive crossbridge dissociation in the presence of magnesium pyrophosphate in skinned rabbit psoas fibers. Biophys. J. 50: 1101-1108.

Annual Report of the Arthritis and Rheumatism Branch
National Institute of Arthritis, Musculoskeletal and Skin Diseases
October 1, 1986 through September 30, 1987

The Branch's professional activities include both clinical and laboratory investigations. The clinical involvement is substantial: During the past year there were some 450 admissions (average length of stay 8 days) accounting for over 3,000 patient-days. Over 2500 follow-up outpatient visits were scheduled including approximately 100 evaluations of new patients. In addition, ARB physicians responded to some 100 requests for consultation at the Clinical Center. The clinical investigations involve the collection and study of tissue and blood specimens as well as trials of various therapeutic regimens, chiefly on patients with systemic lupus erythematosus but also on those with rheumatoid arthritis and polymyositis. In order to assure the smooth functioning of this heavy clinical program, Dr. John H. Klippel serves as Clinical Director for the Branch and Institute.

The laboratory investigations involve studies on animal models of lupus, arthritis and polymyositis as well as fundamental studies at the cellular and molecular level, on various aspects of the immune response and inflammatory processes.

I. Disease Related Studies

A. Rheumatoid Arthritis

1. Pathogenesis

a. Animal Models (48)*

Chronic proliferative and erosive synovitis, as well as heptatic granulomas, can be induced in susceptible rats by systemic administration of aqueous suspensions of cell wall fragments from selected bacteria, such as group A, B or C streptococci and Lactobacillus casei. The arthritis resembles human rheumatoid arthritis in its clinical, pathological and immunological characteristics. The development of acute and chronic disease is a direct consequence of the deposition and persistence of cell wall fragments in hepatic and synovial tissues, primarily in Kupfer cells and endothelial cells, respectively. Comparative studies in athymic-euthymic and cyclosporin A treated/control rats have demonstrated that the early acute phases of disease are independent of the thymus and T-lymphocytes, whereas the chronic phases are thymus-dependent. Chronic disease is characterized by tissue infiltration with lymphocytes, whereas the chronic phases are thymus-dependent. Chronic disease is characterized by tissue infiltration with lymphocytes bearing T helper/inducer cell surface markers. Interestingly, bone and cartilage destruction appears to be mediated by a "transformed" appearing fibroblast-like synoviocyte. Proliferation, and bone destruction mediated

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*Numbers in parentheses refer to last digits of project number, e.g. Z01 AR 41048

by these cells is driven by mononuclear cell derived cytokines. The destructive process is inhibited by cyclosporin A and the retinoid, 4-hydroxyphenyl retinamide. The development of disease is paralleled by enhanced tissue expression of class II-major histocompatibility antigens. Enhanced expression of these antigens is not observed in resistant rat strains. This animal model provides a powerful tool to investigate mechanisms that regulate susceptibility to chronic inflammation, as well as mechanisms involved in tissue destruction such as exhibited in rheumatoid arthritis. New findings include electromicroscopic examination of the hepatic granulomas that occur in the syndrome. The cellular changes were correlated with the production of interleukins 1,2 and 3 and of prostaglandin PGE_2.

b. Human Disease (66)

Synovial tissues and peripheral blood mononuclear cells are obtained from patients with active chronic synovitis, such as rheumatoid arthritis. The analysis of these samples suggests that conditions such as rheumatoid arthritis comprise an imunopathologic spectrum. Patients at the polar extremes of this spectrum display consistent phenotypic and functional characteristics. An anergic subpopulation of patients with rheumatoid arthritis has been compared with a clinically similar nonanergic group. Peripheral blood mononuclear cells from anergic-patients in general exhibited lower T helper/inducer to T suppressor lymphocyte ratios and increased frequencies of HLA-DR bearing T lymphocytes than those from nonanergic patients. Moreover, the peripheral blood mononuclear cells from these patients spontaneously produce high levels of rheumatoid factor. In contrast, synovial tissues from anergic patients in general had high intensity T helper/inducer lymphocyte infiltration, while tissues from the nonanergic group were, in general, sparsely infiltrated by lymphocytes. Moreover, synovial mast cell numbers were closely correlated with the intensity of T cell infiltration, i.e., the more dense the T-cell infiltration, the more numerous were the mast cells. Data also suggested that these phenotypic differences are also reflected by cytokine secretion (e.g., T lymphocyte derived chemotactic factor is produced in abundance by synovium from anergic, but not nonanergic patients). These findings provide insight into pathogenic mechanisms and may result in improved therapeutic procedures.

2. Therapeutic trials

a. Cyclosporin (72)

The efficacy and toxicity of daily, oral cyclosporin A (CSA) in patients with active rheumatoid arthritis is being evaluated in a 6 month double-blind study of low dose (1mg/kg/day) versus high dose (10mg/kg/day) CSA. Toxicity is monitored weekly for the first 2 months and then every 2 weeks until 6 months.

Disease activity is assessed on a bi-monthly basis. Patients who do not develop at least a 40% improvement in disease activity at 6 months are dropped from the study, while those with a 40% or greater response continue for an additional 6 months of drug therapy. The effects of CSA on various measures of T lymphocyte function in peripheral blood and within the synovium is studied in all patients.

The enrollment and 6 month evaluation phases of the trial have been completed. Thirty one patients participated in the study. The principal clinical findings include a) evidence that high dose CSA, but not low dose CSA, results in significant improvement in multiple measures of disease activity and b) both high dose and low dose CSA are associated with a high frequency of toxicity, particularly renal toxicity. Clinical improvements correlated with the finding of no *in vitro* proliferative response of peripheral blood mononuclear cells to a battery of soluble recall antigens (anergy). The anergic status was reversed during treatment with CSA.

b. Methotrexate (73)

The efficacy of low-dose, intermittent oral methotrexate in the treatment of patients with rheumatoid arthritis has been documented by several recent randomized, controlled trials. The mechanism of action of low-dose methotrexate in rheumatoid arthritis has yet to be defined. However, the prompt relapse of the disease upon discontinuing the drug suggests a primary antiinflammatory as opposed to a basic disease modifying action. Moreover, the long term effects of chronic low-dose methotrexate particularly on hepatic and pulmonary function are of considerable concern.

High-dose methotrexate with leucovorin rescue used primarily in the treatment of malignancies has well defined antiinflmmatory and immunosuppressive effects. These properties are likely to be synergistic in the treatment of rheumatoid arthritis. This pilot study will evaluate the effects of intensive intravenous high-dose methotrexate with leucovorin rescue on the synovitis of rheumatoid arthritis. The duration of observed clinical effects will be monitored for an additional four months beyond treatment to detect potential modification of the underlying disease. Quantitation of lymphocyte subpopulations and measures of lymphocyte function will be studied throughout the trail to determine changes induced by high-dose methotrexate.

Five patients with severe, treatment refractory rheumatoid arthritis were treated with high dose intravenous methotrexate (500 mg/m2) followed by leucovorin (50 mg/m^2). Four courses of chemotherapy were given over a 2

month interval. At the end of the final course, there was a 50% or greater improvement in joint swelling and pain indices and morning stiffness in all patients. Clinical responses persisted for 6 to 14 weeks post therapy. High dose methotrexate-leucovorin was associated with a significant reduction in DR antigen expression on peripheral Leu 2, Leu 3 and Leu 4 lymphocytes.

B. Systemic Lupus Erythematosus (SLE)

1. Pathogenesis

a. Animal models (20)

Autoimmunity in New Zealand mice has a genetic and immune basis; however, expression of severity can be modified by viral and other environmental factors as well as sex hormones. The immune factors are complex. These include excessive B-cell activation and abnormal T cell function.

Previous and recent observations lead to the following conclusions.

1. Polyclonal B cell activation is an underlying common feature of murine lupus.
2. Study of autoimmune mice indicates that there are different cellular bases for SLE. This may lead to different treatment for different patients in the future.
3. Environmental agents can trigger SLE in susceptible individuals that would otherwise not get it. Immune enhancers or even certain foods could trigger or accelerate disease.
4. The genetic basis for autoimmunity is becoming much better established. The studies of B-cell defective mice point to potential genetic or monoclonal antibody manipulations in humans with autoimmune diseases.
5. The differential oncogene expression indicates the heterogeneous basis for SLE in different mice. The association of ras with autoimmunity is the first correlate of gene expression and lupus.

b. Human disease (23)

Patients with SLE have excessive B cell proliferation. They have increased proliferation of bone marrow stem cells and circulating B cell precursors. Disease activity is characterized by a switch from just proliferation to differentiation and immunoglobulin synthesis. In some patients the T cell regulation of B cell functions is abnormal. In an attempt to better understand cell-cell interactions, normals were studied for the capacity of T cells to respond to autologous non-T cells. IL 2

production by T4+ cells was necessary for the proliferation of T8+ (suppressor) cells. This function is subnormal in patients with SLE.

Monoclonal antibodies were used to study subpopulations of SLE patients. One subset, with a reduced ratio of helper/suppressor cells, was characterized clinically by renal disease, thrombocytopenia, leukopenia and early age of onset of disease. Another subset had a high ratio and a clinical picture which included muscle and lung disease, Sicca syndrome, lymphadenopathy and CNS disease. Thus, SLE may be be a single disease but a group of syndromes with different genetic and cellular bases.

The more rapidly progressive autoimmune lymphoproliferate disorder, angioimmunoblastic lymphadenopathy, is characterized by markedly effective helper T cells which drive B cells to proliferate and differentiate. The B cells themselves are normal in this variant.

Proto-oncogene expression is abnormal in patients with SLE. There is a significant increase in expression characteristic of activated B cells.

2. Therapeutic studies

a. Animal studies (22)

A diet enriched in a polyunsaturated fatty acid, eicosapentaenoic acid, markedly retards the development of autoimmunity in (NZB x NZW)F1 females. This works even after the onset of disease. Human trials are being carried out.

Cyclophosphamide is effective treatment in a variety of autoimmune strains unrelated to the cellular basis of illness or the nature (B or T cell) of the proliferating cells. This therapy is effective against the lymphadenopathy of MRL-lpr/lpr and C3H/HeJ-gld/gld mice and the coronary artery disease of BXSB and (NZW x BXSB)F_1 mice as well as the renal disease of most examples of murine lupus. Cyclophosphamide markedly reduced the elevated myb RNA in MRL.lpr/lpr mice lymph nodes and increases the very low thymic myb expression.

Cyclosporin A, a drug which interferes with T cell function, is effective treatment for the arthritis, nephritis, and lymphoproliferation of MRL-lpr/lpr mice.

Treatment of (NZB x NZW)F^1 and MRL-lpr/lpr mice with TNF is effective when started either early in life or after the onset of disease.

b. Human disease (40)

Patients with the diffuse forms of systemic lupus erythematosus nephritis previously entered into therapeutic trials continue to be evaluated. Long-term studies suggest that the degree of scarring on the renal biopsy may provide important prognostic information. The subset of patients with a mild degree of scarring responds to oral azathioprine or oral cyclophosphamide when added to low doses of corticosteroids. Patients with a greater degree of scarring are not effectively treated with those drug programs, but may respond to intravenous boluses of cyclophosphamide. The relative effectiveness and toxicity of various drug programs continues to be systematically evaluated. Long-term followup of patients with lupus nephritis demonstrate clear-cut benefit of immunosuppressive-cytotoxic drugs over conventional corticosteroid therapy. The benefit was greatest with cyclophosphamide. The differences are highly significant, but only are apparent when followup is substantially beyond the 5 year point. Intermittent boluses of cyclophosphamide are at least as effective as oral cyclophosphamide and are much less toxic.

C. Polymyositis

1. Etiology/Pathogenesis (69)

Characteristic antibodies which react with normal tissues (autoantibodies) develop in patients with a number of diseases and in some animals. Considerable evidence suggests that some of these antibodies arise because of specific interactions between an exogenous agent (virus, bacterium, drug) and the sites to which the antibody binds. A search is underway to determine whether certain picornaviridiae can be implicated in the causation of inflammatory myositis and the characteristic myositis related antibodies which react with amino acid tRNA synthetases.

Using hybridization methods virus persistence up to at least four weeks has been found in the tissues of mice infected with EMC-221A. Its persistence is not uniformly associated with persistence of viral nucleic acid. There is evidence for the persistence of viral nucleic acid in locations from which living virus can no longer be recovered.

So far, in a small group of muscle biopsies from children with dermatomyositis, we have not found persistence of viral nucleic acid using probes for both the 5' and 3' ends of Coxsackie B viruses. Studies with more biopsies and from our own, now extensive, collection of biopsies from adults will continue using these and other probes.

In studies of the autoantibodies directed to histidyl tRNA synthetase associated with myositis, we have found that all sera recognize identical epitopes of the purified enzyme.

Two variants of encephalomyocarditis virus have been shown to accept an amino acid in a standard charging assay. Attempts to study the location of the reaction on the viral genome are underway.

2. Therapeutic trials (71)

Severe polymyositis or dermatomyositis which responds poorly to corticosteroids may be difficult to treat. Because of anecdotal reports suggesting efficacy, we are comparing plasmpaheresis and leukapheresis with a sham procedure. Of 22 patients randomized in the apheresis trial, so far 6 have clearly improved. An interim blinded data analysis is underway to determine whether or not there is a significant difference among the 3 treatment groups.

Patients who fail to respond will be offered pulse intravenous cyclophosphamide. Starting in the summer of 1987, patients who qualify will be randomized to receive either intravenous methotrexate with leukovorin rescue or a combination of methotrexate and azathioprine.

II. Fundamental Studies (25)

The receptor for immunoglobulin E (IgE) mediates reactions of considerable medical interest; in addition, it serves as a more general model system for effector systems activated by imunoglobulins. The basic aim of these studies is to understand the early events triggered by this receptor in molecular terms. For this purpose studies are pursued for determining the structure of the receptor, exploring the early events it activates and ultimately attempting to understand the structural-functional relationships. During the past year the following new results were obtained: 1. Completion of compositional analyses (including biosynthetic incorporation studies) have provided rigorous criteria for defining the alpha, beta and gamma subunits of the receptor, 2. A cDNA that codes for the alpha chain has been isolated and sequenced and considerable progress has been made in isolating the cDNA for the beta chain, 3. Monoclonal antibodies to the alpha and beta chains have been prepared and used to examine unresolved questions regarding the receptor, 4. Preparation of cytoplasts have been successful and these retain substantial receptor-mediated hydrolysis of phosphoinositides (PI), 5. Further study of PI breakdown by intact cells has shown that in addition to the previously recognized Ca^{2+}-dependent PI breakdown, the tumor cells with which the work is performed also have a Ca^{2+}-independent breakdown. This occurs very early and leads to the formation of the active 1,4,5 inositol trisphosphate. The latter produces a subsequent mobilization of internal stores of Ca^{2}. Evidence has been developed that upon activation of the receptors, protein kinase C is recruited to the plasma membrane.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41020-20 ARB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Pathogenesis of Autoimmunity in Mice with SLE-like Illness

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

Alfred D. Steinberg, Chief, Cellular Immunology Section, NIAMS
Michael F. Seldin, Medical Staff Fellow, Cellular Immunology Section, NIAMS
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John D. Mountz, Medical Staff Fellow, Cellular Immunology Section, NIAMS
William C. Gause, Staff Fellow, Cellular Immunology Section, NIAMS
Arthur Krieg, Medical Staff Fellow, Cellular Immunology Section, NIAMS
Dennis M. Klinman, Med. Staff Fellow and LeeAnn Tracy,Quest Researcher, CIS,NIAMS

COOPERATING UNITS (*if any*)

Foreign: None

LAB/BRANCH

Arthritis and Rheumatism

SECTION

Cellular Immunology

INSTITUTE AND LOCATION

NIAMS - Bldg. 10, Rm. 9N-218 Bethesda, MD. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	2	3

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Autoimmunity in New Zealand mice has a genetic and immune basis; however, severity of expression can be modified by viral and other environmental factors as well as sex hormones. The immune factors are complex. These include excessive B-cell activation and abnormal T cell function.

A single gene, xid, prevents disease in congenic NZB and (NZB x NZW(F$_1$ and BXSB mice, and markedly retards disease in MRL-lpr/lpr mice. BXSB mice have a Y chromosome-linked gene which accelerate autoimmunity.in autoimmune mice, but does not cause it in non-autoimmune mice.

Studies of NZB, BXSB, MRL-lpr/lpr and normal mice show that the repertoires of their B cells are very similar suggesting that B cell activation in these mice is induced by polyclonal activation.

Different autoimmune mice have increased expression of different cellular oncogenes. Lpr/lpr and gld/gld mice have increased myb and T cell receptor (Beta gene) mRNA. The xid gene prevents increased myc and ras expression characteristic of NZB and BXSB mice, suggesting that that oncogene is associated with B cell activation and autoantibody production. The studies of gene expression in lpr/lpr mice have pointed to a new pathway for T cell maturation.

Project Description:

Objectives

Several strains of mice spontaneously develop autoimmune diseases resembling human systemic lupus erythematosus. Early in life they are immunologically hyperactive, become relatively resistant to tolerance induction, and gradually develop antinuclear and other autoantibodies followed by clinical disease. We believe that a detailed understanding of the genetic, cellular and biochemical basis for disease in these mice will contribute to the understanding of a variety of immune mediated diseases.

Methods Employed

1. Assays for antibodies to RNA, DNA and other ligands using ^{14}C-labeled or ^{3}H-labeled ligands and the ammonium sulfate precipitation or solid phase binding assay. The latter has also been adapted to the ELISA technique. Assay of antibody formting cells to a number of antigens by a modified ELISA assay.

2. Immunization of animals with a variety of antigens with and without protein carries or adjuvants.

3. Neonatal thymectomy by midline incision and a suction catheter.

4. Transfer of spleen, bone marrow and thymus cells into recipients to evaluate the functional properites of the different cell types.

5. Study of the graft versus host disease (GVH) inducing properties of lymphoid cells using the Simonsen spleen weight assay as well as the induction of autoantibodies by GVH.

6. Separation of lymphoid cells functionally (corticosteroid, anti-T cell sera, PNA) and physically (use of monoclonal antibodies to subsets).

7. Evaluation of subpopulations of T-cells and of B-cells using the fluorescence activated cell sorter.

8. Castration of male and female mice at various ages and treatment with sex hormones by injection or by implantation of silastic tubes containing crystaline hormones.

9. Chronic injection of polyclonal immune activators with induction or acceleration of autoimmunity.

10. Analysis of low molecular weight cytokines produced by cells of the immune system. This includes induction of receptors for thesecytokines on appropriate target cells. Study of the production and regulation of both the cytokines and their receptors in autoimmune mice.

11. Northern blot analyses. Isolation of poly A^+ mRNA from lymphoid organs or cell subset or cell clones and probing with known probes or probes we produce by subtraction hybridization.

12. In situ hybridization using riboprobes.

13. Southern blot analyses using probes to known markers on distal chromosome 1 of the mouse to home in on gld in [(Mus Spretus x C3H-gld/gld) x C3H/gld/gld] backcross mice.

Major Findings

1. The CBA/N X-linked gene (xid) is completely expressed in congenic mice including such autoimmune congenic mice bearing xid as NZB.xid, NZW.xid, (NZB x NZW)F_1.xid, BXSB.xid, (NZB x BXSB)F_1.xid. Such mice fail to make anti-DNA, anti T cell antibodies, and anti-RBC antibodies. They are protected from renal and cardiac disease and premature death.

2. NZB.xid congenic mice, which do not have autoantibodies and which do not get sick, manifest the T cell defects seen in NZB mice. This result suggests that the T cell defects do not result from the B cell hyperactivity.

3. Consistent with point 2, MRL-lpr/lpr.xid and C3H/HeJ-gld/gld.xid mice have a marked reduction in autoantibody production but unchanged T cell lymphadenopathy.

4. Mice not destined to develop overt clinical autoimmune disease develop rapidly progresive autoimmune disease and premature death when administered polyclonal B cell activators.

5. T cell lines can be established from MRL-lpr/lpr spleens. These lines can produce IL 2, interferon, and IL 4 in vitro and respond to stimulation by Ia^+ non-T cells. The IL 4 and interferon can help B cells to differentiate into Ig secreting cells. The growth of these lines is inhibited by pre-cultured macophages plus anti-Ia antibodies.

6. Sera from NZB mice will allow a subimmunogenic dose of SRBC to be antigenic in normal mice Therefore, an immune enhancer may contribute to autoimmunity.

7. Autoimmune mice manifest differential oncogene expression. Lpr/lpr and gld/gld mice have increased myb and raf mRNA.

8. The xid gene prevents ras and myc expression but not myb.

9. The precentage of B cells reactive with DNA, RBC, T cells, mayosin, actin, transferrin, ovalbumin, and TNP-KLH is very similar in autoimmune and non-autoimmune strains. Therefore, the increased numbers of Ig secreting cells represents polyclonal B cell activation.

Significance to Bio-medical Research and the Program of the Institute

1. Polyclonal B cell activation is an underlying common feature of murine lupus.

2. Study of autoimmune mice indicates that there are different cellular bases for SLE. This may lead to different treatment for different patients in the future.

3. Environmental agents can trigger SLE in susceptible individuals that would otherwise not get it. Immune enhancers or even certain foods could trigger or accelerate disease.

4. The genetic basis for autoimmunity is becoming much better established. The studies of B-cell defective mice point to potential genetic or monoclonal antibody manipulations in humans with autoimmune diseases.

5. The differential oncogene expression indicates the heterogeneous basis for SLE in different mice. The association of *ras* with autoimmunity is the first correlate of gene expression and lupus.

Proposed Course

The role of genetic factors will be further investigated. This will include further study of congenic and recombinant inbred lines. The ultimate goal is to isolate and study the genes involved, identify the gene products, and determine their mechanisms of action. A program of breeding (*Mus Spretus* x (C3H-*gld*/*gld*) x C3H-*gld*/*gld* will lead to isolation of the *gld* gene.

Publications:

1. Miller, M.L., Cowdery, J.S., Laskin, C.A., and Steinberg, A.D.: Studies of tolerance in autoimmune-prone mice, In, *Mucosal Immunity and Infection of Mocosal Surfaces*, ed., W. Strober, M. Lamon and J. McGhee, Oxford University Press, 1986.

2. Scribner, C.L., Seldin, M.F., Santoro, T.J., Smith, H.R., and Steinberg, A.D.: Studies of the genetic bases of murine lupus. I. Medelian analyses of background genes, accelerating genes, inducing genes, and modifying genes. In, *New Horizons in Animal Models of Autoimmune Disease*. Eds. Masahisa Kyogoku, Japan, 1986.

3. Mountz, J.D., Gause, W.C., Siegel, J.N., Seldin, M.F., Cohen, D.I., and Steinberg, A.D.: Studies of the genetic basis of murine lupus. II. Molecular analyses of differential gene expression. In, *New Horizons in Animal Models of Autoimmune Disease*, Eds. Masahisa Kyogoku, Japan, 1986.

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5. Klinman, D.M., and Steinberg, A.D.: Idiotypy and Autoimmunity, Arthritis Rheum. 29:697-705, 1986.

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7. Mountz, J.D., Huppi, K.E., Seldin, M.F., Mushinski, J.F., and Steinberg, A.D.: T cell receptor expression in autoimmune mice. J. Immunol. 137:1029-1036, 1986.

8. Steinberg, A.D., Klinman, D.M., Kastner, D.L., Seldin, M.F., Gause, W.C., Scribner, C.L., Britten, J.L., Siegel, J.N., and Mountz, J.D.: Genetic and molecular genetic studies of murine and human lupus. Proceedings of First International Symposium on SLE, J. Rheumatol. (Supplement), 1986.

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10. Seldin, M.F., Reeves, J.P., Scribner, C.L., Roths, J.B., Davidson, W.F., Morse, H.C. III, and Steinberg, A.D.: Effect of *xid* on autoimmune C3H-*gld*/*gld* mice. Cell Immunol. 107:249-255, 1987.

11. Scribner, C.L., Hansen, C.T., Klinman, D.M., Steinberg, A.D.: The interaction of the *xid* and *me* genes. J. Immunol. 138:3611-3617, 1987.

12. Nelson, J.L., and Steinberg, A.D.: Sex Steriods, Autoimmunity, and Autoimmune Diseases Chapter 6 in Hormones and Immunity ed. I. Berczi MTP Press Ltd., Lancaster, 1987 pp 93-119.

13. Kedar, I., and Steinberg, A.D.: Epitope specific anti-Ia^k antibodies acts specific probes for functional epitopes on Ia^k. European J. Immunol. (in press).

14. Klinman, D.M., and Steinberg, A.D.: Novel Elisa and Elisa spot assays used to quantitate B cells and serum antibodies specific for T-cell and Bromelated mouse red blood cell autoantigens. J. Immunol. Methods. (in press).

15. Klinman, D.M., Steinberg, A.D.: Systemic autoimmune disease arises from polyclonal B cell activation. J. Exp. Med. 165:1755-1760, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 41021-19 ARB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Antinuclear Antibodies in Spontaneous and Drup-induced SLE and Other Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Alfred D. Steinberg, Chief, Cellular Immunology Section, NIAMS
Curtis, L. Scribner, Medical Staff Fellow, Cellular Immunology Section, NIAMS
Dennis M. Klinman, Senior Medical Staff Fellow, Cellular Immunology Section, NIAMS
Lee Ann Tracy, Guest Researcher, Cellular Immunology Section, NIAMS

COOPERATING UNITS *(if any)*
Foreign: NONE

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Cellular Immunology

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	3	2

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Z01 AM 41021-19 ARB

Antibodies to nucleic acids are important diagnostically and pathogenetically in systemic lupus. Patients with active SLE have an increase in DNA in immune complexes. Such DNA is enriched in guanine (G) and cytosine (C). Low molecular weight DNA enriched in G-C is released from SLE leukocytes. This DNA may be important in the pathogenesis of SLE. Genes favoring the production of anti-DNA may include those which lead to the release of immunogenic DNA.

In mice a subset of B cells is responsible for anti-DNA production. In NZB mice, B cells can transfer anti-DNA to congenic NZB.xid recipients. This transfer is prevented by treating either donors animals or recipients with anti-Ia antibodies. Similar approaches to human disease are being sought.

In autoimmune mice, polyclonal B cell activation, rather than specific immunization, is the major contributor to anti-DNA hyperproduction. Patients with SLE have greater repertoire differences than mice. The differences are in IgG but not IgM autoantibodies. In these individuals specific immunization or cross-reactive immunization may also play a critical role in autoantibody production.

Project Description:

Objectives

Study the characteristics of a variety of antinuclear antibodies in human and murine "autoimmune disease". Determine genetic contributions to immune responses to nucleic acid and related antigens.

Methods Employed

Farr ammonium sulfate precipitation assay using radiolabeled ligands. Standard precipitin reaction using labeled and unlabeled nucleic acids or nuclear extracts. Hemagglutination, complement fixation and immunodiffusion assays. Standard immunization procedures. Solid phase enzyme linked immunoadsorbent assay (ELISA) using enzyme-linked antibodies to mouse immunoglobulin or classes of immunoglobulins. Adaptation of the ELISA assay to study of individual antibody secreting cells.

Major Findings

1. Lymphocytes of patients with SLE, but not normals, release low molecular weight DNA. This DNA had an increase in the bases guanine and cytosine. The G-C rich DNA is especially immunogenic in mice and a human hybridoma recognizes it specifically. This DNA may be an important source of immunogenic DNA and of DNA in injurious immune complexes.

2. Mice which spontaneously produce anti-DNA utilize only a small subset of B cells to do so. It has the phenotype Ly 1^+, Thy 1^-, Lyb 5+.

3. B cells from NZB mice transfer anti-DNA production to congenic NZB.*xid* mice. This is prevented by treating either donor animals or recipients *in vivo* with anti-Ia antibodies.

4. The anti-DNA contribution to hypergammaglobulinemia in autoimmune mouse strains is proportional suggesting that polyclonal activation is the critical impetus to anti-DNA in these mice.

Significance to Bio-medical Research and the Program of the Institute

Genes for high and low responsiveness to DNA may be one of the genetic factors in the development of autoimmunity in SLE. One of these genes may regulate the release of G-C rich low molecular weight DNA from lymphocytes, DNA which is both immunogenic and present in injurious immune complexes. In murine lupus, a single gene, *xid*, prevents anti-DNA.

Proposed Course

Further define the genetics of the antibody response to DNA and try to correlate with other immune abnormalities. Define the role of anti-idiotype antibodies in the regulation of anti-DNA production. Further study subpopulations of patients with a high incidence of autoimmunity. Attempt *in*

vitro production of and suppression of anti-DNA. Determine if a unique B cell sub-set is responsible for anti-DNA in humans.

Publications:

1. Steinberg, A.D., Triem, K.H., Smith, H.R., Laskin, C.A., Rosenberg, Y.J., Klinman, D.M., Mushinski, J.F., and Mountz, J.D.: Studies of the effects of Y chromosome factors on the expression of autoimmune disease. Ann. N. Y. Acad. Sci. 475:200-218, 1986.

2. Scribner, C.L., Seldin, M.F., Santoro, T.J., Smith, H.R., and Steinberg, A.D.: Studies of the genetic bases of murine lupus. I. Mendelian analyses of background genes, accelerating genes, inducing genes, and modifying genes. In, *New Horizons in Animal Models of Autoimmune Disease*. Eds. Masahisa Kyogoku, Japan, 1986.

3. Seldin, M.F., Reeves, J.P., Scribner, C.L., Roths, J.B., Davidson, W.F., Morse, H.C. III, and Steinberg, A.D.: Effect of *xid* on autoimmune C3H-*gld*/*gld* mice. Cell Immunol. 107:249-255, 1987.

4. Scribner, C.L., Hansen, C.T., Klinman, D.M., and Steinberg, A.D.: The interaction of the *xid* and *me* genes. J. Immunol. 138:3611-3617, 1987.

5. Steinberg, A.D.: Systemic Lupus Erythematosus: Hospital Medicine. (In press).

6. Klinman, D.M., and Steinberg, A.D.: Novel Elisa and Elisa spot assays used to quantitate B cells and serum antibodies specific for T-cell and Bromelated mouse red blood cell autoantigens. J. Immunol. Methods. (In press).

7. Klinman, D.M., and Steinberg, A.D.: Systemic autoimmune disease arises from polyclonal B cell activation. J. Exp. Med. 165:1755-1760, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41022-15 ARB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Therapeutic Studies in Murine Lupus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Alfred D. Steinberg, Chief, Cellular Immunology Section, NIAMS
John D. Mountz, Medical Staff Fellow, Cellular Immunology Section, NIAMS

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Jay Moore, Univ. of Nebraska

Foreign: None

LAB/BRANCH

Arthritis and Rheumatism

SECTION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A diet enriched in a polyunsaturated fatty acid, *eicosapentaenoic acid,* markedly retards the development of autoimmunity in $(NZB \times NZW)F_1$ females. This works even after the onset of disease. Human trials are being carried out.

Cyclophosphamide is effective treatment in a variety of autoimmune strains unrelated to the cellular basis of illness or the nature (B or T cell) of the proliferating cells. This therapy is effective against the lymphadenopathy of MRL-*lpr*/*lpr* and C3H/HeJ-*gld*/*gld* mice and the *coronary artery disease* of BXSB and $(NZW \times BXSB)F_1$ mice as well as the renal disease of most examples of murine lupus. Cyclophosphamide markedly reduces the elevated *myb* RNA in MRL-*lpr*/*lpr* mice lymph nodes and increases the very low thymic *myb* expression.

Cyclosporin A, a drug which interferes with T cell function, is effective treatment for the arthritis, nephritis, and lymphoproliferation of MRL-*lpr*/*lpr* mice.

Treatment of $(NZB \times NZW)F_1$ and MRL-*lpr*/*lpr* mice with TNF is effective when started either early in life or after the onset of disease.

Project Description:

Objectives

$(NZB \times NZW)F_1$ mice spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. NZB mice develop Coomb^{o}s positive hemolytic anemia, membranous nephrosis and excessive lymphoreticular proliferation. MRL/Mp-lpr/lpr and C3H/HeJ-gld/gld mice develop marked lymphadenopathy, antibodies to DNA and glomerulonephritis. BXSB mice and their hybrids with NZW and NZB mice develop degenerative coronary artery disease in addition to glomerulonephritis. These mice are excellent models for comparing different therapeutic programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying more specific immunotherapy.

Methods Employed

1. Comparison of different immunosuppressive and anti-viral drug regimens on the natural history of mice with lupus by study of autoantibodies, proteinuria, renal and coronary pathology and survival.

2. Treatment with immune modulatory cytokines in an attempt to restore normal regulatory functions and improve the natural history.

3. Treatment with androgens.

4. Treatment with dietary modification that ultimately might be of benefit in humans.

5. Apply knowledge of the genetic basis of disease to new approaches to therapy: for example, use of specific monoclonal antibodies.

Major Findings

1. Cyclophosphamide was effective treatment in $(NZB \times NZW)F_1$, MRL-lpr/lpr, BXSB, and $(NZB \times BXSB)F_1$ mice. Autoantibodies, renal disease, coronary artery disease and survival were all improved. Randomizing all of the mice into a single study made it harder to demonstrate efficacy because each strain required its unique dosage and time of initiation of treatment.

2. Cyclophosphamide normalized abnormal proto-oncogene expression in C3H/HeJ-gld/gld and MRL-lpr/lpr mice.

3. MRL/Mp-lpr/lpr mice had amelioration of disease by neonatal thymectomy. They had a decrease in anti-DNA and proteinuria, and a marked increase in survival. Their lymphadenopathy was almost completely prevented. Splenectomy at 2 weeks of age, but not 8 weeks of age was similarly very effective therapy. Poly rI.rC reversed the beneficial effects of thymectomy.

4. A diet enriched in fish oil containing a polyunsaturated fatty acid eicosapentaenoic acid markedly prolonged life in (NZB x NZW)F1 mice when started before or after the onset of the autoimmune process.

5. TNF alpha reduced lymphadenopathy in MRL-lpr/lpr mice and protein uria in (NZB x NZW)F_1 mice. Survival was prolonged in both strains.

6. The xid gene markedly retards the development of autoimmunity in (NZB x NZW)F_1, BXSB, NZB, MRL-lpr/lpr, C3H/HeJ-gld/gld mice.

7. Cyclosporin A markedly prolongs life and reduces the lymphadenopathy of MRL-lpr/lpr mice. It also prevents arthritis and nephritis without reducing anti-DNA, immune complexes, or rheumatoid factor.

Significance to Bio-Medical Research and the Program of the Institute

The mice continue to provide a testing-ground for promising new treatments prior to assessing their effects in patients. Despite the widespread effectiveness of cyclophosphamide, we recognize the difficulty in demonstrating efficacy in heterogeneous populations. We are especially impressed by the possibility that a dietary supplement may be an important adjunct to therapy in SLE. We hope also to be able to exploit the finding that a single gene largely prevents the development of murine lupus.

Proposed Course

Extension of the diet studies to humans. Further analysis of xid effects with an aim toward human application.

Publications:

1. Steinberg, A.D., Klinman, D.M., Rosenberg, Y.J., Honda, M., Smith, H.R., Santoro, T.J., Reeves, J.P., Seldin, M.F., Mushinski, J.F. and Mountz, J.D.: Autoimmune lymphoproliferative disorders: Studies of pathogeneis and approaches therapy. In, Mediators of Immune Regulation and Immunotherapy; eds. S.K. Singhal, Elsevier Science Publishing Co., Inc., London, Ontario, Canada, 1986, pp 213-231.

2. Kedar, I., Rosenberg, Y.J., and Steinberg, A.D.: Growth regulation of transformed T cells by non-activated macrophages: The role of Ia expression. J. Immunol. 136:3166-3171, 1986.

3. Robinson, D.R., Prickett, J.D., Makoul, G.T., Steinberg, A.D., and Colvin, R.B.: Dietary fish oil reduces progression of established renal disease in (NZB x NZW)F_1 mice and delays renal disease in BXSB and MRL/1 strains. Arthritis Rheum. 29:539-546, 1986.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41023-13 ARB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Immune Abnormalities in Patients with Systemic Lupus Erythematosus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Alfred D. Steinberg, Chief, Cellular Immunology Section, NIAMS
Dennis M. Klinman, Medical Staff Fellow, Cellular Immunology Section, NIAMS
LeeAnn Tracy, Guest Researcher, Cellular Immunology Section, NIAMS
Yoshiaki Ishigatsubo, Visiting Fellow, Cellular Immunology Section, NIAMS

COOPERATING UNITS *(if any)*

C. Morimoto, Harvard Medical School

Foreign: None

LAB/BRANCH

Arthritis and Rheumatism

SECTION

Cellular Immunology

INSTITUTE AND LOCATION

NIAMS - Bldg. 10, Rm. 9N-218, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with SLE have excessive B cell proliferation. They have increased proliferation of bone marrow stem cells and circulating B cell precursors. Disease activity is characterized by a switch from just proliferation to differentiation and immunoglobulin synthesis. In some patients the T cell regulation of B cell functions is abnormal. In an attempt to better understand cell-cell interactions, normals were studied for the capacity of T cells to respond to autologous non-T cells. IL 2 production by $T4^+$ cells was necessary for the proliferation of $T8^+$ (suppressor) cells. This function is subnormal in patients with SLE.

Monoclonal antibodies were used to study subpopulations of SLE patients. One subset, with a reduced ratio of helper/suppressor cells, was characterized clinically by renal disease, thrombocytopenia, leukopenia and early age of onset of disease. Another subset had a high ratio and a clinical picture which included muscle and lung disease, Sicca syndrome, lympadenopathy and CNS disease. Thus, SLE may not be a single disease, but a group of syndromes with different genetic and cellular bases.

The more rapidly progressive autoimmune lymphoproliferate disorder, angioimmunoblastic lymphadenopathy is characterized by markedly effective

helper T cells which drive B cells to proliferate and differentiate. The B cells themselves are normal in this variant.

Proto-oncogene expression is abnormal in patients with SLE. There is a significant increase in expression characteristic of activated B cells.

Project Description:

Objectives

To apply the newer understanding of basic immunologic principles, and observations in mice who develop lupus-like diseases, to the study of patients with SLE.

Methods Employed

1. Study of families of patients with SLE or larger groups of patients with a high incidence of SLE. A battery of immunologic measurements would be made in patients.
2. Study of lymphocytes and antibodies to lymphocytes from patients.
    a. Number and distribution of T, B macrophages and null cells, analysis of their surface markers and their responses to mitogens or allogeneic cells.
    b. Study of antibody producing cells in the peripheral blood of patients.
    c. Study of leukocyte cell surface markers by use of monclonal antibodies and flow cytometry.
    d. Use of the technique of flow microfluorometry to separate immune cell subpopulations reactive with specific monoclonal antibodies.
    e. ELISA assay for analysis of antibody produced *in vitro*.
3. Production of monoclonal antibodies to human cells.
4. Molecular genetic techniques for analyzing expression of genes involved in proliferation differentiation.

Major Findings

1. Studies with monoclonal antibodies, which identify T cell subsets, indicate that patients with active SLE may have in abnormal T suppressor/T helper ratios; the abnormal ratio stems from a quantitative defect in helper cells rather than suppressor cells. High suppressor: helper ratios are associated with renal disease, thrombocytopenia, and lymphopenia. In constrast, patients with relatively fewer suppressors than normal tend to have a multisystem disease with muscle disease, lymphadenopathy, the sicca syndrome, and older age of onset of disease. Symptom clusters are also appreciated without the need for T cell phenotyping.
2. *Proto-oncogene* expression is abnormal in SLE patients. Those oncogenes associated with B cell activation have increased expression in patients with active disease, and also in patients with other autoimmune rheumatic diseases.

3. Angioimmunoblastic lymphadenopathy is characterized by excessive helper T cell function with essentially normal B cells.

4. Sera from patients with SLE have repertoires of binding to a panel of ligands much more divergent than different autoimmune mouse strains. The major differences were in IgG not IgM antibodies.

Significance to Bio-medical Research and the Program of the Institute

The studies to date suggest that patients with SLE have B cell hyperactivity. Both genetic factors and environmental exposure determine which B cell clones will be expanded. Loss of suppressor and helper cells in human SLE is associated with (a) disease activity, and (b) anti-T-cell antibodies. These defects preferentially occur during disease activity, indicating that the abnormalities are not permanent, and that appropriate specific prevention may be possible.

The association of clinical features with the ratio of suppressor to helper cells suggests that SLE may have a different cellular basis in different patients. Thus, SLE may not be a disease entity, but a clinical syndrome with different causes. This would direct further study of pathogenesis and therapy at patient subgroups.

Proposed Course

Continue the studies intitiated. Further analyze differential gene expression at the molecular level. Try to analyze genetics in families with multiple members.

Publications:

1. Klinman, D.M., Mushinski, J.F., Honda, M., Ishigatsubo, Y., Mountz, J.D., Raveche, E.S., and Steinberg, A.D.: Oncogene expression in autoimmune and normal peripheral blood mononuclear cells. J. Exp. Med. 163:1292-1307, 1986.

2. Steinberg, A.D., Klinman, D.M., Rosenberg, Y.J., Honda, M., Smith, H.R., Santoro, T.J., Reeves, J.P., Seldin, M.F., Mushinski, J.F., and Mountz, J.D.: Autoimmune lymphoproliferative disorders: Studies of pathogeneis and approaches to therapy. In, Mediators of Immune Regulation and Immunotherapy; eds. S.K. Singhal, Elsevier Science Publishing Co., Inc., London, Ontario, Canada, 1986, pp 213-231.

3. Klinman, D.M., and Steinberg, A.D.: Autoimmunity, In, Systemic Lupus Erythematosus, Ed. R.G. Lahita. John Wiley and Sons, Inc., 1986.

4. Klinman, D.M., and Steinberg, A.D.: Idiotypy and Autoimmunity, Arthritis Rheum. 29:697-705, 1986.

5. Raveche, E.S., and Steinberg, A.D.: Studies of Autoimmunity. In, Handbook of Experimental Immunol; 4th Edition, eds. D.M. Weir, L.A. Herzenberg, C.C. Blackwell and L.A. Herzenberg. Blackwell Scientific Publications Ltd., Edenburgh, U.K. 1986.

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7. Steinberg, A.D., Klinman, D.M., Kastner, D.L., Seldin, M.F., Gause, W.C., Scribner, C.L., Britten, J.L., Siegel, J.N., and Mountz, J.D.: Genetic and molecular genetic studies of murine and human lupus. Proceedings of First International Symposium on SLE, J. Rheumatol. (Supplement), 1986.

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9. Smolen, J.S., Klippel, J.H., Penner, E., Reichlin, M., Steinberg, A.D., Chused, T.M., Scherak, O., Graninger, W., Hartter, E., Zielinski, C.C., Wolf, A., Davie, R., Mann, D.L., and Mayr, W.R., HLA-DR antigens in systemic lupus erythematosus: Association with specificity of autoantibody responses to nuclear antigens. Ann. Rheum. Dis. 46:457-462, 1987.

10. Morimoto, C., Steinberg, A.D., Levitan, N.L., Hagan, M., Daley, J., Levine, H., Schlossman, S.F.: A defect in immunoregulatory T cell subsets in SLE patients demonstrated with anti-2H4 antibody. J. Clin. Invest. 79:762-768, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 41025-16 ARB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the cell surface receptor for IgE

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
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Arthritis and Rheumatism Branch

SECTION
Chemical Immunology

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 8	PROFESSIONAL: 8	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The receptor for immunoglobulin E (IgE) mediates reactions of considerable medical interest; in addition it serves as a more general model system for effector systems activated by immunoglobulins. Our basic aim is to understand the early events triggered by this receptor in molecular terms. For this purpose we are detrmining the structure of the receptor, exploring the early events it activates and ultimately will attempt to understand the structural-functional relationships. During the past year the following new results were obtained: 1. Completion of our compositional analyses (including biosynthetic incorporation studies) have provided rigorous criteria for definiing the alpha, beta and gamma subunits of the receptor, 2. A cDNA that codes for the alpha chain has been isolated and sequenced and considerable progress has been made in isolating the cDNA for the beta chain, 3. Monoclonal antibodies to the alpha and beta chains have been prepared and used to examine unresolved questions regarding the receptor, 4. Preparation of cytoplasts has been successful and these retain substantial receptor-mediated hydrolysis of phosphoinositides (PI), 5. Further study of PI breakdown by intact cells has shown that in addition to the previously recognized Ca-dependent PI breakdown, the tumor cells with which we work also have a Ca-independent PI breakdown. This occurs very early and leads to the formation of the active 1,4,5 inositol trisphosphate. The latter produces a subsequent mobilization of internal stores of Ca, 6. Evidence has been developed that upon activation of the receptors, protein kinase C is recruited to the membrane.

Project Description:

Objectives:

IgE is a unique class of immunoglobulin that plays a predominant role in the pathophysiologic events associated with a variety of allergic phenomena. The IgE mediates its functions by binding to plasma membrane receptors on mast cells and related cells. The binding itself is not known to produce changes; however, if the cell bound IgE becomes aggregated (e.g by binding to a multivalent antigen), rapid degranulation of the cell ensues. It is now known that the critical initiating event is aggregation of the receptor to which the IgE is bound. Our objective is to understand the immediate molecular sequelae of this aggregation since this may have implications for therapeutic manipulation of this system as well as other antibody-mediated cell perturbations.

Methods Employed

Molecular biological analysis has been set up in the laboratory and is being used to isolate, sequence and express genes for the receptor for IgE.

Major Findings

A. Structural studies

1. Compositional analysis and peptide mapping

Compositional analysis was completed and combined with biosynthetic studies. These now provide rigorous structural criteria for the individual chains. These data plus the sequence data that is becoming available will rigorously define the size and multiplicity of the receptors subunits. The data will also provide significant criteria for judging the correctness of the genes that are being isolated for the subunits since there are few other criteria so far available to characterize the subunits.

2. Gene cloning studies

Gene cloning studies were initiated and have already achieved significant success. Since the subunits all have blocked N-terminal residues, tryptic peptides had to be isolated. This was accomplished for the alpha subunit of the receptor

and an oligonucleotide probe based on such a sequence hybridized with several colonies in a cDNA library prepared from fractionated mRNA of RBL cells. When analyzed, the cloned cDNA showed an open reading frame that codes for a putative 23 residue leader sequence plus a 27 kDa protein - almost exactly what the molecular weight data had predicted for the peptide mol. wt. for alpha. The sequence has revealed the following:

a) It predicts an amino acid composition in good agreement with the composition detrmined directly.

b) It predicts sequences of five other peptides we subsequently isolated from digests of isolated alpha chains.

c) The sequence predicts that alpha is a transmembrane protein with an extracellular portion of 180 residues containing two immunoglobulin-like domains. It predicts a 20 residue transmembrane domain containing a charged amino acid (Asp) and a 27 residue highly-basic cytoplasmic tail.

d) The extracellular and putative transmembrane segment show substantial homology to the sequence recently reported for the IgG binding Fc-receptor of macrophages.

The gene codes for a protein that reacts with a polyclonal antibody prepared to the receptor. In addition, anti-peptide antibodies that were prepared from peptides based on the sequence of the gene appear to react with proteins produced by cells transfected with the gene and most significantly with purified alpha chains of the receptor.

Peptides have now been prepared from the beta subunit and one of these was used to prepare an oligonucleotide probe. This, in turn, has given positive results with the cDNA library and a cloned cDNA is now being sequenced with preliminary results looking very favorable.

Work is in progress to obtain peptides from the

gamma chain so that the gene for this chain can be isolated likewise.

3. Monoclonal antibodies

It has been difficult to prepare monoclonal antibodies that will react with the subunits on Western blots. Some success has recently been achieved for the alpha chain as well as the beta chain. The properties of these antibodies is currently being explored.

B. Functional studies

The goal of our functional studies is two-fold:

First to identify early biochemical events mediated by the receptor and second, to try to see if such events can be demonstrated on preprations of plasma membranes.

We have focussed on the receptor-mediated hydrolysis of phosphoinositides. Recent results are as follows:

1. Whereas earlier studies by another group had suggested that unlike other cells, RBL cells would only exhibit stimulated PI turnover in the presence of Ca^{2+}, we found that such turnover did occur even in the absence of extracellular Ca^{2+}. This is important since it would be difficult to assess membrane preparations if the physiological Ca^{2+} gradient would have to be maintained.

2. Whereas earlier studies had indicated that RBL cells produce principally the inactive 1,3,4 inositol trisphosphate isomer, our recent studies shw that in fact the cells produce an immediate Ca^{2+}-independent burst of the active isomer: 1,4,5 inositol trisphosphate.

3. Whereas earlier studies had failed to observe a sequential rise in PI hydrolysis followed by a mobilization of internal stores, we have now been able to observe such a sequence if the RBL cells are studied at very early times after stimulation.

4. Despite exhaustive efforts we have not yet been able to observe receptor mediated PI turnover on membrane preparations. However, we have been successful in preparing active cytoplasts. Such prepartions have a plasma membrane, normal amounts of cytoplasmic proteins but are markedly depleted

of cytoplasmic organelles such as mitochondria, granules and of course, nuclei. We have carefully characterized such preparations both with respect to their constituents and with respect to their ability to hydrolyze PIs in response to receptor crosslinking. Interestingly, these preparations show similar amounts of activity as intact cells in the absence of Ca^{2+}. We are currently assessing whether the cytoplasts will provide a useful intermediate for the production of active preparations of membranes.

5. Studies on protein kinase C

We have explored the RBL cells to see if they show receptor mediated translocation of protein kinase C to the plasma membrane. We found that in the presence of phorbol esters and ionophore, such translocation is substantial. Similarly, when the IgE receptors are aggregated, a small but reproducible translocation is observed and this is maintained as long as the receptors remain aggregated. However although there is an excellent correlation between such translocation and secretion (exocytosis), this is not the case if the protein kinase C is depleted by exposing the cells to high doses of phorbol esters for 18-24 hrs. In this case the cells fail to secrete in response to PMA and ionophore but still show substantial release in response to aggregation of IgE receptors. This suggests that the translocation of protein kinase C may be an incidental rather than necessary event in the secretory pathway stimulated by receptor aggregation.

Significance to Bio-Medical Research and the Program of the Institute

The IgE-Mast cell system is of significance in the pathophysiology of allergy, parasitic infections, and inflammation. It is also an experimentally convenient system to study mechanisms of antigen-induced antibody-mediated cellular reactions in general. Finally, it is an excellent model of the study of exocytosis. So far, we are continuing to make good progress in the elucidation of the molecular mechanisms of this system. Knowledge of these may eventually permit rational intervention in situations where the system contributes to pathology.

Proposed Coures:

The principal projects for the next year will be:

1) Complete isolation and sequencing of cDNA for beta subunit of receptor.

2) Prepare peptides of gamma subunit, prepare oligonucleotide probe based on peptide sequence and isolate cDNA coding for this subunit.

3) Explore by Northern blotting and use of monoclonal antibodies to beta and gamma whether either the RBL cells or other cells have these or related peptides associated with other receptors.

4) Cotransfect the genes for the three subunits to obtain expression of native receptors in eukaryotic cells. See if such receptors can trigger PI turnover in such cells. If so, begin study of what the critical elements of the structure are by gene modifications.

5) Continue attempts to obtain active membrane preparations.

Honors: Dr. Metzgr was elected as a Fellow of the American Association for the Advancement of Science.

Publications

1. Metzger, H., Alcaraz, G., Kinet, J-P., and Quarto, R. the receptor for immunoglobulin E as a membrane protein. Biochemical J. 51:59-67, 1986.

2. Metzger, H. The Mast Cell Receptor for Immunoglobulin E: Prospects for Therapy, in Proc. XII Int. Cong. Allerg. Clin. Imunol. (ed:) C.V. Mosby Co., St. Louis, MO 1986, pp. 308-311.

3. Grasberger, B., Minton, A.P., DeLisi, C., and Metzger, H. Interaction between proteins localized in membranes. Proc. Natl. Acad. Sci. 83:6258-6262, 1986.

4. Quarto, R., and Metzger, H. The receptor for immunoglobulin E: Examination for kinase activity and as a substrate for kinases. Molec. Immunol. 23:1215-1223, 1986.

5. Alcaraz, G., Kinet, J.-P., Liu, T.-Y., and Metzger, H. Further characterization of the receptor with high affinity for Immunoglobulin E. Biochemistry 26, 2569-2575, 1987.

6. Kinet, J.-P., Metzger, H., Hakimi, J., and Kochan, J. Isolation of a presumptive cDNA coding for the alpha subunit

of the receptor with high affinity for Immunoglobulin E. Biochemistry, in press.

7. Pribluda, V.A., & Metzger, H. Ca independent phosphoinositide breakdown in rat basophilic leukemia cells. J. Biol. Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41040-15 ARB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of various cytotoxic drug programs in diffuse lupus neprhitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Alfred D. Steinberg, Chief, Cellular Immunology Section, NIAMS
Paul H. Plotz, Senior Investigator, ARB, NIAMS
John M. Klippel, Senior Investigator, ARB, NIAMS
Ronald L. Wilder, Senior Investigator, ARB, NIAMS

COOPERATING UNITS *(if any)*

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Howard A. Austin, Attending Nephrologist, Clinical Center

Foreign: None

LAB/BRANCH

Arthritis and Rheumatis

SECTION

Cellular Immunology

INSTITUTE AND LOCATION

NIAMS - Bldg. 10, Rm. 9N-218, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Z01 AM 41040-15 ARB

Patients with the diffuse forms of systemic lupus erythematosus nephritis previously entered into therapeutic trials continue to be evaluated. Long-term studies suggest that the degree of scarring on the renal biopsy may provide important prognostic information. The subset of patients with a mild degree of scarring responds to oral azathioprine or oral cyclophosphamide when added to low doses of corticosteroids. Patients with a greater degree of scarring are not effectively treated with those drug programs, but may respond to intravenous boluses of cyclophosphamide. The relative effectiveness and toxicity of various drug programs continues to be systematically evaluated. Long-term follow-up of patients with lupus nephritis demonstrate clear-cut benefit of immunosuppressive-cytotoxic drugs over conventional corticosteroid therapy. The benefit was greatest with cyclophosphamide. The differences are highly significant, but only are apparent when follow-up is substantially beyond the 5 year point. Intermittent boluses of cyclophosphamide are at least as effective as oral cyclophosphamide and are much less toxic.

Project Description:

Objectives

The Arthritis and Rheumatism Branch has a major interest in the management of the nephritis of systemic lupus erythematosus (SLE). Renal failure is the most feared complication in the disease. The renal damage is largely in the glomeruli. It has been suggested that drugs capable of altering the antibody response, "immunosuppressives", would reduce the formation of antibody and thus of antigen-antibody complexes. The objectives of this study is to assess the efficacy and toxicity of various immunosuppressive drug programs in SLE nephritis.

Methods Employed

Patients with systemic lupus erythematosus and active nephritis have been entered into several trials. All patients are repeatedly reevaluated with a battery of renal function tests, serological studies, urinalyses and, after two years in the trial, by renal biopsy when possible. Treatment failures can be withdrawn according to protocol. Long-term follow-up of patients previously admitted continues. Our latest protocol involves randomizing patients to receive either bolus cyclophosphamide or bolus methylprednisolone or both.

Major Findings

1. Long-term follow-up shows that separately oral cyclophosphamide (CY) and oral azathioprine (AZ) are only of benefit to a subgroup of patients with minimal chronic changes on biopsy.

2. Young patients (8-23 years of age) are of greater risk of developing renal failure than are older (>24 years of age) patients with SLE and nephritis.

3. The data suggest that oral CY is less effective and more toxic than bolus CY.

4. Long-term follow-up demonstrates a substantial and highly significant benefit of cyclophosphamide over corticosteroids. This was only apparent beyond the 5 year follow-up point.

Significance to Bio-Medical Research and the Program of the Institute

These studies are relied upon by physicians concerned with SLE all over the world. We have been one of very few units able to speak from the hard data base of controlled studies.

Proposed Course

We will continue to follow these patients on their assigned programs.

We will enter new patients into a trial of monthly therapy. Patients receive intravenous cyclophosphamide intravenous corticosteroids, or both to treat accelerated or histologically active renal disease in patients with SLE.

Publications:

1. Steinberg, A.D.: Current management of renal disease in patients with systemic lupus erythematosus and approaches to the future. In, Biologically Based Immunomodulators in the Therapy of Rheumatic Diseases. Eds. S. Pincus, D. Pisetsky, L. Rosenwasser, Elsevier Publishing Co., New York, 1986. pp 39-56.

2. Steinberg, A.D.: The treatment of lupus nephritis. Kidney International 30: 769-787, 1986.

3. Lupus Nephritis. Ann. Intern. Med. 106: 79-94, 1987.

4. Steinberg, A.D.: Systemic Lupus Erythematosus: Hospital Medicine. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 41048-08 ARB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Bacterial Cell Wall-Induced Arthritis and Hepatic Granuloma Formation in the Rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R. L. Wilder, Sr. Investigator, ARB/NIAMS
A. Roberts, Sr. Investigator LC/NCI
R. Lafyatis Med. Staff Fellow, ARB/NIAMS
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E. Remmers Staff Fellow, ARB/NIAMS
S.M. Wahl, Sr. Investigator LMI/NIDK
J.B. Allen Biochemist LMI/NIDR
M. Sporn Chief LC/NCI

COOPERATING UNITS *(if any)*
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Laboratory of Chemoprevntion, NCI
Foreign: None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMS/NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Chronic proliferative and erosive synovitis, as well as hepatic granulomas, can be induced in susceptible rats by systemic administration of aqueous suspensions of cell wall fragments from selected bacteria, such as group A, B or C streptococci and Lactobacillus casei. The arthritis resembles human rheumatoid arthritis in its clinical, pathological and immunological characteristics. The development of acute and chronic disease is a direct consequence of the deposition and persistence of cell wall fragments in hepatic and synovial tissues, primarily Kupffer and endothelial cells, respectively. Comparative studies in athymic/euthymic and cyclosporin A treated/control rats have demonstrated that the early acute phases of disease are independent of the thymus and T-lymphocytes; whereas, the chronic phases are thymus-dependent. Chronic disease is characterized by tissue infiltration with lymphocytes bearing T helper/inducer cell surface markers. Interestingly, bone and cartilage destruction appears to be mediated by a "transformed" appearing fibroblast-like synoviocyte. Proliferation and bone destruction mediated by these cells is driven by mononuclear cell derived cytokines. The destructive process is inhibited by cyclosporin A and the retinoid, 4-hydroxyphenyl retinamide. The development of disease is paralleled by enhanced tissue expression of class II-major histocompatibility antigens. Enhanced expression of these antigens is not observed in resistant rat strains. This animal model provides a powerful tool to investigate mechanisms that regulate susceptibility to chronic inflammation, as well as mechanisms involved in tissue destruction such as exhibited in rheumatoid arthritis.

Project Description:

Objectives

Bacterial cell wall-induced arthritis is an experimental animal model of chronic proliferative and erosive synovitis that is induced by systemic administration of biodegradation resistant bacterial cell wall fragments in aqueous solution. The clinical, pathological, biochemical and immunological features of the model closely resemble rheumatoid arthritis. Studies in progress have the following goals:

A. To define the properties of bacterial cell walls that facilitate the development of arthritis.

B. To characterize the host genetic factors that regulate susceptibility to develop arthritis.

C. To better define pathophysiologic processes by characterizing differences between high and low responder inbred rat strains.

D. To define mechanisms leading to tissue injury and destruction.

E. To develop techniques to modulate the process.

Major Findings

Background/Earlier Findings:

I. Clinical/Pathological Features

  A. Articular stages - biphasic

    1. Acute erythema and swelling of peripheral joints may be observed within 24 hours of systemic administration cell walls. Histologically, the acute inflammation is characterized by edema, fibrin deposition in joint space and synovium, and acute cellular infiltration. This phase peaks at about day 3 and subsides over next week.

    2. An exacerbation of swelling is observed beginning 14-21 days after injection that persists for months. Histologically it is characterized by synovial lining cell proliferation and villus formation, and infiltration of the subsynovium with lymphocytes and macrophages. Marginal erosions develop at the junctions of synovium with bone and cartilage. The primary cell as these sites is a "transformed" appearing fibroblast-like cell. Ultimately, the joints are destroyed and develop fibrous ankylosis.

  B. Hepatic Disease

1. Acute inflammation in the liver develops within 24 hours. This process may ultimately progress to the non-caseating granulomas and hepatic fibrosis.

C. Other tissues - mononuclear hyperplasia is prominent in the spleen and other lymphoid tissues.

II. Cell Wall Factors

A. Properties of disease inducing cell walls

1. Cell wall fragments (peptidoglycan with an intact group specific carbohydrate) from Group A, B or C are arthritogenic.

2. Cell wall fragments from selected species of lactobacilli are arthritogenic.

3. All arthritogenic cell walls persist in tissue for prolonged periods of time (T 1/2 are on the order of months).

4. All arthritogenic cell walls defined up to the present time have been resistant to lysozyme degradation and contain large amounts of rhamnose in their group specific carbohydrate.

5. Fragment size determines the time after injection that arthritis develops and determines the duration of disease.

6. Cell walls deposit in synovial tissues of peripheral joints after systemic administration. Cell walls also deposit in spleen, liver and bone marrow.

III. Role of the Immune System in the Pathologic Process

A. Studies in athymic/euthymic rats and cyclosporin A (CSA) treated/non-treated LEW/N rats.

1. All rats (i.e, athymic and CSA-treated as well) develop acute arthritis coincident with the deposition of cell walls in blood vessels in synovial tissues. Similar processes are observed in the liver. These results provide strong evidence that the acute disease is independent of the thymus and thymus-derived lymphocytes.

2. The chronic proliferative and erosive phases of disease do not develop in athymic or cyclosporin A treated rats, thus providing evidence that this stage is thymus-

dependent. This stage is characterized by heavy infiltration in affected tissue of W3/13+, W3/25+, OX-19+, OX8- T helper/inducer cells. These cells do not infiltrate the liver and joints in the athymic rats or CSA-treated LEW/N rats.

3. Enhanced expression of Ia antigens, parallelling disease severity, is observed in the diseased tissues.

B. Activated T-lymphocytes can be readily isolated from synovium and liver of animals with chronic lesions.

IV. Tissue Injury/Destruction

A. Bone and cartilage erosion is associated with the pronounced proliferation of a "transformed" appearing fibroblast-like cell. Immunohistochemical staining, in situ, indicates that these cells do not express markers in common with T-lymphocytes and macrophages (i.e., they lack W3/13, W3/25, OX8, Ia, as well as various non-specific esterases). Staining for cytoskeletal markers indicates that the cell is of mesenchymal origin. These cells have a "transformed" phenotype in culture and form "tumor" nodules when injected into athymic mice.

B. The proliferation of the fibroblast-like cell and the associated bone and cartilage destruction are markedly inhibited in vivo by both CSA and the synthetic retinoid, 4-hydroxyphenyl retinamide. CSA in vitro does not affect these cells indicating that its effect is indirect (probably inhibits T-cell production of cytokines that act upon this cell).

V. Secreted Products (Cytokines)

A. Since the tissue destruction appears to be mediated by fibroblast-like cells, but the development of chronic inflammation appears dependent upon the thymus and T-lymphocytes, investigation into the mechanisms by which the T-lymphocytes regulate synovial fibroblast growth and activation is in progress. We have demonstrated that the inflammed synovial tissues, particularly the fibroblast-like cell, are major producers of collagenase and prostaglandin E_2.

B. Synovial mononuclear cells spontaneously secrete interleukin 1.

C. Resting splenic or peripheral blood mononuclear cells do not make these cytokines but can be stimulated to do so.

VI. Other Immunologic Features

A. Cell wall-injected rats develop severe acquired immunologic dysfunction. For example, splenic and lymphoid mononuclear cells lose their capacity to proliferate in response to mitogens and to secrete interleukin 2. Available data indicate that this dysfunction is mediated by a macrophage derived suppressor factor that is not a prostaglandin.

B. Cell wall injected rats produce large amounts of immunoglobulin.

VII. Host Susceptibility

A. Genetic Factors

1. Susceptibility is markedly dependent on genetic background (e.g., LEW/N female rats are particularly susceptible, but F344/N and BUF/N females are not).

2. Incidence and severity of disease are influenced by several gene loci, including non-MHC loci (not an all-or-none phenomena).

3. Athymic congenic LEW and F344 rats have been developed. The LEW.rnu/rnu develops severe acute but no chronic arthritis. The F344.rnu/rnu develops minimal or no disease. These data indicate that regulatory mechanisms independent of the thymus are also involved in this model.

B. Gender is a major factor in selected rat strains, but not in others.

1. LEW/N females are highly susceptible, but LEW/N males are not.

2. Sex linked effects are hormonally mediated (castration or estrogen administration to males markedly exacerbates disease).

C. Resistance to chronic disease in F344/N, BUF/N, LER/N and CAR/N females is not due to a failure of cell walls deposit in synovial and hepatic tissues. Cell walls distribute similarly in these rat strains, but chronic disease does not develop. These strains exhibit striking differences in qualitative levels of MHC-related Ia antigen in the liver and synovial tissues following cell wall injection (i.e., Ia expression in disease susceptible LEW/N is high, but low in disease resistant and MHC-compatible F344/N, CAR/N and LER/N). These observations provide potential molecular level insights into the mechanisms regulating disease susceptibility.

New Findings:

A. Hepatic granulomas

1. Ultrastructural analysis of the lesions by electron microscopy showed that the early lesions are composed of small lymphocytes surrounded by phagocytic cells. Eosinophils and mast cells were prominent. As the lesions matured, monocytes-macrophages at various stages of differentiation were noted. Fibroblasts were frequently noted juxtaposed to macrophages.

2. The spontaneous production of multiple cytokines and inflammatory mediators was demonsrated. These included IL1, IL2, IL3 and PGE_2. The kinetics of their production correlated with the evolution of the hepatic granulomas.

B. Synovial Tissues

Studies similar to those with the hepatic granulomas are in progress but not yet complete. It is apparent that multiple mononuclear and nonmononuclear cell derived growth and inhibitory factors regulate the development of the synoviocyte proliferation and invasion into bone and cartilage.

Significance to Biomedical Research and Program of the Institute

Peptidoglycan-containing bacterial cell wall fragments have received increasing attention in recent years as possible pathogenetic agents in some cases of chronic synovitis. This experimental animal model provides a powerful tool to explore the pathogenesis of chronic proliferative and erosive arthritis. We have defined parameters of both the host (cell wall distribution, inflammatory mechanisms, role of T-lymphocytes and various cytokines, etc.) and the bacteria (type, resistance to biodegradation, fragment size) which influence the development of arthritis. In particular, this model continues to provide new insight into possible mechanisms of reactive type polyarthritis in man. New research opportunities continue to emerge.

Proposed Course:

The genetic, immunologic and pathophysiologic characterization of the host parameters that determine arthritis susceptibility with emphasis on the MHC, are continuing. Additional emphasis is being placed on the role of the immune system in regulating connective tissue growth and differentiation.

Publications

1. Allen, J.B., Yocum, D.E. Wahl, S.M., Wilder, R.L. Inhibition by cyclosporin A of streptococcal cell wall-induced hepatic granulomas in LEW/N rats. Annals of the New York Academy of Sciences 465:351-361, 1986.

2. Wilder, R.L. Streptococcal cell wall-induced polyarthritis in rats, In "CRC Handbook of Animal Models for the Rheumatic Diseases, R. Greenwald, H. Diamond (Ed.), in press.

3. Wahl, S.M., Allen, J.B., Dougherty, S. Evequoz, V., Pluznik, D.H. Wilder, R.L., Hand, A.R., Wahl, L.M. T lymphocyte-dependent evolution of bacterial cell wall-induced hepatic granulomas. J. Immunol. 137:21:99-2209, 1986.

4. Allen, J.B., Wilder, R.L. Variable severity and Ia antigen expression in streptococcal-cell-wall- induced hepatic granulomas in rats. Infection and Immunity 55:674-679, 1987.

5. Wilder, R.L., Allen, J.B., Hansen, C. Thuymus-dependent and independent-regulation of Ia antigen expression in situ by cells, in the synovium of rats with streptococcal cell wall-induced arthritis. Journal of Clinical Investigation 79:1160-1171, 1987.

6. Wilder, R.L. Streptococcal cell wall-induced arthritis in rats: an overview: In The Proceedings of the Second International Congress on Inflammation", in press.

7. Wilder, R.L., Animal Models of Reactive Arthritis. In "Infection and Reactive Arthritis, In press.

8. Wilder, R.L. Proinflammatory microbial products as etiologic agents in inflammatory arthritis. In "The Pathogenesis of Chronic Inflammatory Arthritis', in press.

Several additional manuscripts are being prepared.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AR 41051-07 ARB

PERIOD COVERED

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Natural History and in situ Organization of the Receptors for IgE

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project Terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41066-05 ARB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Synovial Tissue from Patients w/RA & Related Conditions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R.L. Wilder, Senior Investigator, ARB/NIAMS

COOPERATING UNITS *(if any)*

Laboratory of Microbial Immunology, NIDR Foreign: None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Diseases

INSTITUTE AND LOCATION

NIAMS/NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Synovial tissues and peripheral blood mononuclear cells are obtained from patients with active chronic synovitis, such as rheumatoid arthritis. The analysis of these samples suggests that conditions, such as rheumatoid arthritis, comprise an immunopathologic spectrum. Patients at the polar extremes of this spectrum display consistent phenotypic and functional characteristics. An anergic subpopulation of patients with rheumatoid arthritis has been compared with a clinically similar nonanergic group. Peripheral blood mononuclear cells from anergic patients, in general, exhibited lower T helper/inducer to T suppressor lymphocyte ratios and increased frequencies of HLA-DR bearing T lymphocytes and natural killer cells than those from nonanergic patients. Moreover, the peripheral blood mononuclear cells from these patients compared to nonanergic patients spontaneously produced high levels of rheumatoid factor. In contrast, synovial tissues from anergic patients, in general, had high intensity T helper/incuder lymphocyte infiltration, while the tissues from the nonanergic group was, in general, sparsely infiltrated by lymphocytes. Moreover, synovial mast cell numbers were closely correlated with the intensity of T cell infiltration, i.e., the more dense the T-cell infiltration, the more numerous were the mast cells. Data also suggested that these phenotypic differences are also reflected by cytokine secretion (e.g., T lymphocyte derived chemotactic factor is produced in abundance by synovium from anergic, but not from nonanergic patients). These findings provide insight into pathogenic mechanisms and may result in improved therapeutic procedures.

Project Description:

Objectives:

The inner surface of the capsule of diarthrodial joints is lined by a delicate vascular connective tissue known as the synovial membrane. It consists of one or two layers of synovial lining cells (synoviocytes) overlaying an areolar or fibrous connective tissue. In conditions such as rheumatoid arthritis (RA), the surface layer of the membrane is markedly thickened and the deeper areolar tissue of the membrane is filled by infiltrating T lymphocytes, macrophages and plasma cells, producing a picture typical of chronic inflammation. The purpose of this study is to further characterize this inflammatory tissue by study of tissue biopsy specimens and to relate these characteristics to other body compartments such as blood.

The objectives of the study are to obtain tissue for the following:

1. Characterization of the infiltrating mononuclear cells. We are analyzing frozen sections of synovial tissue with a battery of well-characterized monoclonal antibodies. In conjunction with studies of peripheral blood mononuclear cells, these analyses provide insight into lymphocuyte and monocyte migration into these tissues and their role in tissue injury.

2. In vitro culture studies. We are examining these tissues and cells isolated from these tissues for production of a variety of fibroblast proliferation factors, monocyte chemotactic factors, etc., and attempting to characterize the regulatory mechanisms controlling their production.

3. Identification of etiologic factors. We are examining the tissues for etiologic agents, particularly bacterial cell wall peptidoglycans. Collaborative studies are in progress searching for various viruses.

Methods Employed

1. Patient Selection

    a. Patients with possible, probable, definite or classical rheumatoid arthritis are the primary focus of these studies, but in certain instances patients with other etiologies of synovitis are asked to participate. The patients are informed of the purpose of the study, the state of activity of their disease, and that the results of the study will not modify their present therapy. They also are told that, although the purpose of the biopsy is not therapeutic, the concurrent simple removal of synovial fluid may be of transient benefit in itself.

2. Criteria for Biopsy

a. A significantly swollen knee in a patient with the appropriate underlying disease is the primary criterion, but patients with significantly swollen shoulders, elbows wrists, ankles, olecranon bursa or even smaller joints are not excluded if appropriate tissue cannot be obtained from a knee. Patients with any of the following are excluded:

(1) Abnormal bleeding or clotting tests
(2) Platelet count <100,000
(3) Sepsis

Repeat biopsies are requested, but no more than two procedures are done. Selected patients with significantly inflamed joints, are given an intraarticular injectin of steroids after biopsy.

3. Technique of Biopsy

a. Arthroscopy with biopsy under direct visualization

Previous Findings

1. Synovial tissue from clinically active rheumatoid arthritis exhibits a wide spectrum of immunohistologic findings. Specimens from individual patients can be segregated into groups (A,B and I) with contrasting features.

2. Group A specimens have diffuse lymphocytic infiltration, which are predominantly T cells. The T cell subsets bearing OKT4 or Leu 3 surface antigens tend to be the primary infiltrating cell compared to T cells bearing OKT8 2 surface antigens. HLA-Dr bearing T cells are abundant.

3. Group B specimens exhibit sparse lymphocyte infiltration, but have prominent fibrin deposition. The few T lymphocytes present tend to be primarily those bearing the OKT8 or Leu 3 surface antigens, in contrst to Group A specimens.

4. Group A patients often have an increased frequency of HLA-Dr. bearing T lymphocytes in the peripheral blood (5-25%), in contrst to Group B patients. These HLA-Dr bearing T cells appear to be primarily associated with OKT8/Leu 3 antigen bearing subset.

6. Group I specimens tend to exhibit features intermediate to those of Groups A and B.

7. Anergic patients, in general, have synovial tissues with group A features while nonanergic patients tend, in general to exhibit group B features, although overlap is clearly apparent.

8. All tissue specimens, regardless of group assignment, spontaneously produce significant quantities of fibroblast

proliferative factors. Group A tissue specimens produce substantially more monocyte chemotactic factor than group B specimens.

9. Peripheral blood mononuclear cell subset composition differed significantly in anergic and nonanergic patients. Anergic patients had decreased frequencies of OKT4 and decreased frequencies of OKT8-bearing T lymphocutes and lower ratios of OKT4/OKT8 than nonanergic patients. Anergic patients had higher frequencies of HLA-Dr bearing T lymphocytes (12-35%) than nonanergic patients and normal individuals (<10%). The HLA-Dr bearing cells were expressed on both the OKT4 and OKT8 bearing T lymphocyte subsets. but the frequency was higher in the OKT8 fraction.

New Findings:

1. Strong positive correlations were found between numbers of mast cells per cubic millimeter of synovial tissue and the following synovial tissue parameters: the intensity of lymphocyte infiltration, the intensity of LEU-3A (T-helper/inducer) or LEU-1 (PAN T-cell) infiltration and the intensity of plasma infiltration. A strong negative correlation was found between the synovial mast cell count and sublining fibrin deposition. Intraarticular corticosteroids were shown to reduce mast cell counts 67-96%, whereas oral methotrexate therapy had no apparent effect on mast cell counts.

2. Peripheral blood mononuclear cells from 30 active RA patients on no remittive drugs were characterized with regard to spontaneous and pokeweed mitogen (PWM)-stimulated immunoglobulin production and plaque-forming cells (PFC's), proliferative responses to soluble recall antigens and spontaneous IgM rheumatoid factor production (IgM-RF).

   Rheumatoid spontaneous total (IgG and IgM and IgA) IgPFC's were higher than those of normal controls when assayed after 7 days in culture. Spontaneous and PWM-stimulated IGM-PFC's in contrast were significantly less than normal regardless of when assayed. Spontaneous synthesis of IgM-RF was detected in 56% of the RA patients but the absolute amounts varied widely. Spontaneous IgM-RF production by RA PBMC was associated with low or absent spontaneous IgM-PFC production. Moreover, a strong association was found between high level spontaneous IgM-RF production and depressed proliferative responses to soluble recall antigens. These patients have previously been shown to have synovial biopsy specimens that are intensely infiltrated with T-lymphocytes. These findings suggest that subgroups of rheumatoid patients that show distinct cellular and humoral abnormalities can be identified.

Significance

An understanding of chronic synovitis, the primary mechanism by which joint destruction is produced in diseases such as rheumatoid arthritis, requires knowledge about the types of cells infiltrating the inflammed tissues, their differentiation state, the mechanisms regulating their activity, etc. It is also important to understand how other body compartments (e.g., the blood) relate to the diseased synovium. Our studies address these questions and provide insight into potential pathogenetic mechanisms and therapeutic approaches.

Proposed Course

Studies are continuing. A search for potential microbial etiologic agents is in progress.

Publications

1. Wilder, R.L. Cellular immune mechanisms in rheumatoid arthritis. EOS 8:711-14, 1987.

2. Malone, D.G., Wilder, R.L., Saavedia-Delgado, A. Metcalfe, D.D. Mast cell numbers in Rheumatoid Synovial Tissues. Arthritis Rheumatism 30:130-136, 1987.

3. Boling, E.P., Ohishi, T., Wahl, S.M., Misiti, J., Wistar, R., Wilder, R.L. Humoral immune function in severe, active rheumatoid arthritis. Clinical Immunology and Immunopathology 43:185-194, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AR 41069 04 ARB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The role of viruses in the induction of autoantibodies in humans and mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. P. H. Plotz, Sr. Investigator, ARB/NIAMS
F.W. Miller, Med. Staff Fellow, ARB/NIAMS
T. Biswas, Visiting Fellow, ARB/NIAMS
M. Cronin, Medical Staff Fellow, ARB/NIAMS

COOPERATING UNITS *(if any)*
Laboratory of Oral Medicine, NIDR   Foreign: None

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects   ☐ (b) Human tissues   ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Characteristic antibodies which react with normal tissues (autoantibodies) develop in patients with a number of diseases and in some animals. Considerable evidence suggests that some of these antibodies arise because of specific interactions between an exogenous agent (virus, bacterium, drug) and the sites to which the antibody binds. A search is underway to determine whether certain picornaviridiae can be implicated in the causation of inflammatory myositis and the characteristic myositis related antibodies which react with amino acid tRNA synthetases.

Project Description:

Objectives:

1. To determine whether there is direct or indirect evidence for picornavirus infection in human polymyositis.

2. To study in detail the pathogenesis of myositis produced in mice by picornaviruses, encephalomyocarditis (EMC 221A) or Mengovirus.

Methods:

Objective 1.

A. To search for the presence of autoantibodies in patients with myositis by a variety of techniques, focusing especially on antibodies to amino acid t-RNA synthetases. Methods include immunoprecipitation of radiolabelled cell extracts, inhibition of amino acid charging, and a sold phase assay using purified histidyl-t-RNA synthetase. For this latter assay, a new biochemical purification using HPLC has allowed rapid recovery of enzyme purified over 3000-fold.

B. Muscle tissue, blood cells, and plasma have been collected and stored, or cultured, to search for the presence of viruses. When cDNA probes of Coxsackie viruses are obtained, in situ hybridization of will be used to search for viral genome.

Objective 2.

A. Myositis has been induced in adult mice of a variety of strain by EMC-221A. Its natural history, pathology, biochemical abnormalities, and immunology are being studied.

B. Myotropic viral variants are being selected in myoblast culture lines.

C. The interaction of the viral genome with amino acid-tRNA synthetase is being studied.

D. The persistence of virus in infected mice is being studied using virus culture, slot blot hybridization of RNA extracted from tissues, and *in situ* hybridization.

E. Methods to greatly increase the sensitivity of viral detection are being developed.

Major Findings:

1. Using hybridization methods virus persistence up to at least four weeks has been found in the tissues of mice infected with EMC-221A. Its persistence is not uniformly associated with

persistence of viral nuceic acid. There is evidence for the persistence of viral nucleic acid in locations from which living virus can no longer be recovered.

2. So far, in a small group of muscle biopsies from children with dermatomyositis, we have not found persistence of viral nucleic acid using probes for both the 5' and 3' ends of Coxsackie B viruses. Studies with more biopsies from children and from our own now extensive collection of biopsies from adults will continue using these and other probes.

3. In studies of the autoantibodies directed to histidyl tRNA synthetase associated with myosiis, we have found that all sera recognize identical epitopes of the purified enzyme.

4. Two variants of encephalomyocarditis virus have been shown to accept an amino acid in a standard charging assay. Attempts to study the location of the reaction on the viral genome and underway.

Significance to Bio-Medical Research and the Program of the Institute:

Direct evidence of a link between a particular viral infection and a particular autoantibody would re-orient thinking about the etiology and pathogenesis of so-called autoimmune disease.

Proposed Course:

Studies to continue

Publications

1. Biswas, T., Miller, F.W., Twitty, S.A., and Plotz, P.H. An efficient methd for enrichment of histidyl-tRNA synthetase (Jo01 antigen) from HeLa cells. J. Immunol Methods 1987, 98:235-241.

2. Biswas, T., Miller, F.W., Takagaki, Y., and Plotz, P.H. An enzyme-linked immunosorbent assay for the detection and quantitation of anti-Jo-1 antibody in human serum. ibid. 1987, 98:243-248.

3. Miller, F., W., Biswas, T., Cronin, M.E., and Plotz, P.H. All sera with anti-Jo-1 antibody recognize the same proteolytic polypeptides of histiduyl-tRNA synthetse (Jo-1 antigen). Arthritis Rheum 1987, 30(suppl): S47.

4. Miller, F.W., Love, L.A., Biswas, T., McClintock, P.R., Notkins, A.L., and Plotz, P.H. Viral and host genetic factors influence encephalomyocarditis viral-induced polymyositis in adult mice. Arthritis Rheum 1987. 30:549-556.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AR 41070 02 ARB

PERIOD COVERED

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Nature of the DNA Anti-DNA Antibodies in Sera of Patients with SLE

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41071 03 ARB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A controlled trial of apheresis in treatment of poly/dermatomyositis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. P.H. Plotz, Sr. Investigator, ARB/NIAMS
M. Cronin Med. Staff Fellow ARB/NIAMS
F.W. Miller, Sr. Staff Fellow ARB/NIAMS

COOPERATING UNITS *(if any)*

Blood Bank, CC, NIH
Dept. of Rehabilitation, CC,NIH;
Dept. Radiology, CC, NIH; MDB/NIDDK
Lab of Oral Medicine, NIDR, NIH

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Section on Connective Tissue Diseases

INSTITUTE AND LOCATION

NIAMS/NIH/Bethesda, MD 20892

TOTAL MAN-YEARS: 2	PROFESSIONAL: 2	OTHER:

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☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Severe polymyositis or dermatomyositis which responds poorly to corticosteroids may be difficult to treat. Because of anecdotal reports suggesting efficacy, we are comparing plasmapheresis and leukapheresis with a sham procedure. Patients who fail to respond will be offered pulse intravenous cyclophosphamide. Starting in the summer of 1987, patients who qualify will be randomized to receive either intravenous methotrexate with leukovorin rescue or a combination of methotrexate and azathioprine.

Project Description:

Objectives:

1. To determine whether plasmapheresis or lymphopheresis improves the course of severe polymyositis or dermatomyositis.

2. To determine whether cyclophosphamide given intravenously improves the course of patients who fail to respond to apheresis.

3. To obtain specimens for studying the pathogenesis of the illness, especially to look for evidence of viral infection.

Methods:

Patients with PM or DM resistant to steroids or in whom severe steroid side-effects are present are randomized to 4 weeks (12 prcedures) of plasmapheresis, lymphapheresis, or a sham procedure. Careful testing is done to determine the effect of therapy. Patients who fail to respond are offered a trial with pulse intravenous cyclophosphamide for six months. Starting in July 1987, patients will be randomized to receive either methotrexate or a combination of methotrexate and azathioprine.

Major Findings:

Of 22 patients randomized in the apheresis trial, so far 6 have clearly improved. An interim blinded data analysis is underway to determine whether or not there is a significant difference among the 3 treatment groups.

Significance to Bio-Medical Research and the Program of the Institute

This is an attempt to improve the therapy of this very serious illness and to allow better study of its pathogenesis.

Publications:

None yet

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AR 41072-03ARB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cyclosporin A in Rheumatoid Arthritis (RA)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: J.H. Klippel, Senior Investigator, ARB/NIAMS
R.L. Wilder, Senior Investigator, ARB/NIAMS
D. Yocum, Guest Staff, ARB/NIAMS
L. Gerber, Chief, Rehab/CC
J. Minor, Pharmacy/CC
C. Yarboro, Nursing/CC
C. Berkebile, Nursing/CC

COOPERATING UNITS *(if any)*
Rehabilitation/CC, Pharmacy/CC, Nursing/CC   Foreign: None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMS/NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The efficacy and toxicity of daily, oral cyclosporin A (CSA) in patients with active rheumatoid arthritis is being evaluated in a 6 month double-blind study of low dose (1mg/kg/day) versus high dose (10mg/kg/day) CSA. Toxicity is monitored weekly for the first 2 months and then every 2 weeks until 6 months. Disease activity is assessed on a bi-monthly basis. Patients who do not develop at least a 40% improvement in disease activity at 6 months are dropped from the study, while those with a 40% or greater response continue for an additional 6 months of drug therapy. The effects of CSA on various measures of T lymphocyte function in peripheral blood and within the synovium is studied in all patients.

Project Description:

Objectives:

Cyclosporin A is an investigational immunomodulatory drug with a preferential action on T lymphocyte function. In rheumatoid arthritis, the T lymphocyte is thought to play a major critical role in acute and chronic synovial inflammation. This randomized double-blind trial will compare the efficacy of high and low dose cyclosporin to reduce the synovitis of rheumatoid arthritis. The effects of the drug on several measures of T lymphocyte function both within the synovium and peripheral blood will be studied on patients participating in the trial.

Methods Employed

Patients with active seropositive rheumatoid arthritis who have failed conventional therapies will be entered into the study. Patients will be randomized to either high dose (10mg/kg/day) or low dose (1mg/kg/day) cyclosporin. Patients will be followed closely for 6 months with objectives assessments of arthritis activity and screening for drug toxicity.

Major Finding

The enrollment and 6 month evaluation phases of the trial have been completed. Thirty one patients participated in the study. The principle clinical findings include a) evidence that high dose CSA, but not low dose CSA, results in significant improvement in multiple measures of disease activity and b) both high dose and low dose CSA are associated with a high frequency of toxicity, particularly renal toxicity. Clinical improvements correlated with the finding of no *in vitro* proliferative response of peripheral blood mononuclear cells to a battery of soluble recall antigens (anergy). The anergic status was reversed during treatment with CSA.

Significance to Biomedical Research and Program of the Institute:

The study will evaluate in a controlled, double-blind manner, a promising new drug for the treatment of rheumatoid arthritis. Patients participating in the trial will also be included in research studies assessing histologic and immune features of rheumatoid arthritis.

Proposed Course:

Clinical date on 14 patients who demonstrated significant clinical improvement in rheumatoid disease activity following 6 months of CSA and continued to receive CSA for a maximum of 12 months is being analyzed. Manuscripts detailing the clinical findings, immunomodulatory effects of CSA, and effects of the drug on renal function are in preparation.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41073-02ARB

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

High dose methotrexate with leucovorin rescue in rheumatoid arthritis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: J.H. Klippel, Senior Investigator, ARB/NIAMS
J.B. Shiroky, LOM/NIDR
B.A. Chabner, DCT/NCI
C.J. Allerga, DCT/NCI
G.Inghirami, KDS/NIDDK

COOPERATING UNITS *(if any)*

DCT/NCI, KDS/NIDDK   Foreign: None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Diseases

INSTITUTE AND LOCATION

NIAMS/NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects   ☐ (b) Human tissues   ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Five patients with severe, treatment refractory rheumatoid arthritis were treated with high dose intravenous methotrexate (500 mg/m^2) followed by leucovorin (50 mg/m^2). Four courses of chemotherapy were given over a 2 month interval. At the end of the final course, there was a 50% or greater improvement in joint swelling and pain indices and morning stiffness in all patients. Clinical responses persisted for 6 to 14 weeks post therapy. High dose methotrexate-leucovorin was associated with a significant reduction in DR antigen expression on peripheral Leu 2, Leu 3 and Leu 4 lymphocytes.

Project Description:

Objectives:

The efficacy of low-dose, intermittent oral methotrexate in the treatment of patients with rheumatoid arthritis has been documented by several recent randomized, controlled trials. The mechanism of action of low-dose methotrexate in rheumatoid arthritis has yet to be defined. However, the prompt relapse of the disease upon discontinuing the drug suggests a primary antiinflammatory as opposed to a basic disease modifying action. Moreover, the long term effects of chronic low-dose methotrexate particularly on hepatic and pulmonary function are of considerable concern.

High-dose methotrexate with leucovorin rescue used primarily in the treatment of malignancies has well defined antiinflammatory and immunosuppressive effects. These properties are likely to be synergistic in the treatment of rheumatoid arthritis. This pilot study will evaluate the effects of intensive intravenous high-dose methotrexate with leucovorin rescue on the synovitis of rheumatoid arthritis. The duration of observed clinical effects will be monitored for an additional four months beyond treatment to detect potential modification of the underlying disease. Quantitation of lymphocyte subpopulations and measures of lymphocyte function will be studied throughout the trial to determine changes induced by high-dose methotrexate.

Methods Employed:

Five patients with active seropositive rheumatoid arthritis who have failed conventional therapies will be entered into this pilot study. Patients will be treated with intravenous methotrexate (500mg/meter square) followed by leucovorin 24 hours afterwards. Serum methotrexate levels will be closely monitored and additional luecovorin and intravenous hydration given if drug levels at 48 hours exceed $5.0x10^{7}$M. Courses of high-dose methotrexate-leucovorin will be repeated every two weeks for a total of 4 doses. Objective assessments of arthritis activity and screening for drug toxicity will be done at biweekly intervals for the first 4 months of the study and then monthly for an additional two months. Fluorescent cytofluorographic analyses will be done on Ficoll purified peripheral blood mononuclear cells prior to treatment, 14 days after the initial dose and two weeks after the final dose.

Significance to Biomedical Research & Programs of the Institutes

This pilot clinical study will investigate a promising experimental therapy for patients with severe, active rheumatoid arthritis. Studies of basic immune function and the changes induced by high-dose, intravenous methotrexate will contribute to

investigations of the immunopathogenesis of rheumatoid arthritis.

Proposed Course:

A manuscript describing this study has been submitted. Consideration is being given to further evaluation of high-dose methotrexate in rheumatoid arthritis or other immune-mediated inflammatory diseases by formal randomized, controlled clinical trials.

Publications: None

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